DETECTION OF MUTATIONS IN NUCLEIC ACID SEQUENCES

The present invention pertains to the fields of molecular biology and diagnostic medicine. More specifically, the invention relates to methods for distinguishing a mutant allele comprising a mutation from a corresponding wild type allele or other allele lacking the specific mutation. For some applications, the method of the invention may be used to distinguish a mutant allele having oncogenic properties, such as an oncogene or proto-oncogene, from an allele lacking the mutation that confers oncogenic properties.

RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No. 60/479,896, filed June 19, 2003. The content of the aforementioned application is hereby expressly incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

Several publications and patent documents are referenced in this application in order to more fully describe the state of the art to which this invention pertains. The disclosure of each of these publications and documents is incorporated by reference herein.

The detection of mutations in DNA is of significant importance in a variety of fields, particularly with regard to diagnostic and prognostic applications. The diagnosis of genetically determined diseases and identification of disease carriers, for example, relies in large part on the identification of DNA mutations associated with the disease in individuals. Indeed, in Northern Europe diseases caused by genetically determined defects may affect 1% of all live births. In some Mediterranean countries, 20% of the population is predicted to have a genetic defect associated with thalassaemia. Moreover, many cancers are known to be associated with or caused by a mutation in a nucleic acid sequence. Such mutations generally involve a substitution of one or more bases, a deletion of one or more bases, or the insertion of one or more bases. A number of methods have been proposed for detecting such mutations. Some of the methods shall now be illustrated.

Conventional methods of gene analysis involve DNA isolation and restriction digestion, gel electrophoresis and Southern DNA blotting, hybridization with radioactive probes and washing, and finally autoradiography. Several days are required for this process. Such methods are the subject of a review by Little in "Genetic Engineering" volume 1, pages 61-102, 1981, Academic Press.

Such methods can be used whether or not the DNA has been accurately sequenced in the region of interest. But this approach has major disadvantages, including the ability to detect point mutations only when present in a restriction enzyme cleavage site, and then only when other cleavage sites for the same enzyme are not present within the general vicinity. These disadvantages have inhibited the use of these techniques for genetic screening in clinical laboratories.

When the DNA sequence in the region of interest is known, it is possible to overcome some of these disadvantages. Conner et al. (Proc. Natl. Acad. Sci. U.S.A., 80, 1983, 278-282) describe a method that does not require the mutation to be located within a restriction enzyme cleavage site. A radioactively-labeled oligonucleotide probe is hybridized to the region of the DNA which includes the possible mutation. The hybridization conditions are empirically determined to optimize differential hybridization of the probe to a nucleic acid sequence with the mutation, as opposed to a nucleic acid sequence lacking the mutation. The length of the probe and the hybridization conditions are also experimentally determined, the optimization of which is difficult and critical for success. The necessity of using a radioactively labeled probe and tedious preliminary steps render this method of limited utility.

DNA (RNA) sequencing may also be used to determine the nucleic acid sequence of a molecule suspected or known to be associated with a disease state or disorder. Although nucleic acid sequencing is very reliable, it is prohibitively expensive and involves multiple procedural steps requiring a high degree of technical expertise for execution.

A DNA chip wherein a number of oligonucleotides are fixed onto a glass surface and selectively hybridized with a substance to be analyzed (e.g., a DNA fragment comprising a mutation) may be used to detect a signal (e.g., a fluorescent signal) that reveals hybridization of the substance to the chip. Comparison of this signal with that produced by hybridization to a "normal substance" (e.g., a DNA fragment which does not comprise the mutation) provides means to estimate the sequence of the substance. The cost of such DNA chips, however, is prohibitive. Moreover, the number of

oligonucleotides fixed onto the chip must be varied according to the substance, which further amplifies methodological expenses.

In the SSCP (Single Strand Conformation Polymorphism) method, double stranded nucleic acid sequence (DNA or RNA) is dissociated into single stranded nucleic acid sequence and the higher-order structure of the single stranded nucleic acid sequence may be determined by, for example, polyacrylamide gel electrophoresis. A specific higher-order structure of a single stranded nucleic acid sequence is dependent on the nucleic acid sequence of the molecule and, thus, a difference of a single nucleotide in a molecule is detectable as a change in the mobility of the molecule in a polyacrylamide gel following electrophoresis. SSCP, therefore, provides a means to estimate the presence/absence of a monobasic substitution. The SSCP method, however, requires detailed analysis of optimal electrophoretic conditions for each sample, a process which is tedious and cumbersome and, therefore, not amenable to high throughput analyses.

In the DGGE (Denaturing Gradient Gel Electrophoresis) method, a sample comprising a PCR (polymerase chain reaction) product is separated by electrophoresis in a polyacrylamide gel with a concentration gradient of a denaturant optimized for comparing the dissociation of different DNA molecules from double stranded to single stranded DNA. A difference of a single base pair may, therefore, be detected using this method. The DGGE method, however, requires detailed analysis of the optimal composition of the electrophoretic gel for each sample, an involved process that is not amenable to high throughput analyses.

In the DHPLC (Denaturing High Performance Liquid Chromatography) method, a sample of double stranded DNA and a standard double stranded DNA (i.e., a double stranded DNA with a known base sequence, such as a wild type DNA) are mixed, thermally denatured to be dissociated into single stranded DNA, and thereafter cooled to to reanneal the single stranded DNA to become double stranded DNA. When a sample includes only double stranded DNA with a standard base sequence, only a homoduplex is formed. When a sample includes a double stranded DNA with a monobasic substitution in addition to the standard double stranded DNA, homoduplexes and heteroduplexes having a mismatch site formed at the site of the substitution are formed. A heteroduplex has a smaller number of hydrogen bonds formed than a homoduplex, as a consequence of the presence of the mismatch. The heteroduplex may, therefore, be detected using high-speed liquid chromatography which can differentiate molecules based on distinct melting temperatures (i.e., T_m: temperature at which 50% of the total concentration of double

stranded DNA is denatured to single stranded DNA). Of note, the T_m of the heteroduplex is lower than that of the homoduplex, and may be distinguished on this basis. The DHPLC method, however, is expensive due to the cost involved with liquid chromatography.

SUMMARY OF THE INVENTION

The present invention is directed to a method for detecting a mutation in a target nucleic acid sequence, said method comprising:

(a) providing a plurality of nucleic acid sequences comprising said target nucleic acid sequence, said target nucleic acid sequence comprising said mutation, wherein said mutation does not produce a restriction endonuclease site;

(b) providing a first primer hybridizable near a first portion of said target nucleic acid sequence comprising said mutation, said first primer comprising a second portion of said target nucleic acid sequence; and a second primer hybridizable to said target nucleic acid sequence, wherein said first and second primer comprise a polymerase chain reaction (PCR) primer pair; wherein the first of the primers of this pair changes the DNA sequence of the resultant PCR amplicon to produce a new restriction endonuclease site at the site of the mutation;

(c) performing a PCR amplification of said plurality of nucleic acid sequences using said PCR primer pair, wherein said PCR amplification produces a target nucleic acid sequence amplicon comprising a site recognizable by a restriction endonuclease, said site comprising the first portion and second portion of said restriction endonuclease site, said first portion and second portion of said restriction endonuclease site operably linked by said PCR amplification;

(d) incubating PCR amplicons produced in step (c) with said restriction endonuclease capable of recognizing said restriction endonuclease site under conditions permissible for said restriction endonuclease activity; and

(e) detecting nucleic acid fragments produced in step (d), wherein detection of a pattern of nucleic acid fragments is indicative of a presence of said target nucleic acid sequence amplicon comprising a site recognizable by said restriction endonuclease,

said pattern of nucleic acid fragments being indicative of a presence of a mutation in said target nucleic acid sequence in said plurality of nucleic acid sequences.

In accordance with the method of the present invention, a target nucleic acid sequence can be an allele associated with a disorder, an oncogene, or a proto-oncogene. Such target nucleic acid sequences are isolated from a biological sample, such as, for example, a patient sample.

In one aspect of the method of the invention, the presence of a mutation in a target nucleic acid sequence is indicative of a disorder. Such disorders include, but are not limited to genetic diseases, metabolic diseases, and hyperproliferative diseases. An example of a hyperproliferative disease diagnosable using the method of the invention is a cancer.

In another aspect of the method of the invention, the presence of a mutation in a target nucleic acid sequence is indicative of a predisposition to a disorder. Such disorders include, but are not limited to genetic diseases, metabolic diseases, and hyperproliferative diseases. A predisposition to a hyperproliferative disease such as a cancer, for example, is determinable using the method of the invention.

In one embodiment, the method of the invention is used to detect a mutation in an oncogene that is an activating point mutation. Such mutations occur in a number of genetic alleles including, but not limited to, a *B-RAF* allele, a *K-RAS* allele, and an *N-RAS* allele.

In another embodiment, the method of the invention is used to detect a mutation in an oncogene that is an inactivating mutation including, but not limited to, a *BRCA* or *EGFR* gene mutation.

In an aspect of the invention, the method is used to detect a *B-RAF* allele encoding a mutated *B-RAF* polypeptide comprising a V599E mutation. In accordance with the method of the invention, a PCR primer pair comprising a first primer SEQ ID NO: 7 and a second primer SEQ ID NO: 8 is used to amplify amplicons from the mutated *B-RAF* allele/template. Such amplicons comprise an Alw26 I restriction endonuclease site. Digestion of amplicons generated from a mutated *B-RAF* allele with Alw26 I produces a pattern of nucleic acid fragments, including fragments of 123 and 37 base pairs. Detection of this diagnostic pattern of nucleic acid fragments provides a positive indicator of the presence of the V599E mutation in the specimen.

In another aspect of the invention, the method is used to detect an *N-RAS* allele encoding a mutated *N-RAS* polypeptide comprising a Q61R mutation. In accordance with

the method of the invention, a PCR primer pair comprising a first primer SEQ ID NO: 9 and a second primer SEQ ID NO: 10 is used to amplify amplicons from the mutated *N-RAS* allele/template. Such amplicons comprise a Bcg I restriction endonuclease site. Digestion of amplicons generated from such a mutated *N-RAS* allele with Bcg I produces a pattern of nucleic acid fragments, including fragments of 168, 34, and 22 base pairs. Detection of this diagnostic pattern of nucleic acid fragments provides a positive indicator of the presence of the Q61R mutation in the specimen.

In another aspect of the invention, the method is used to detect an *N-RAS* allele encoding a mutated *N-RAS* polypeptide comprising a Q61K mutation. In accordance with the method of the invention, a PCR primer pair comprising a first primer SEQ ID NO: 11 and a second primer SEQ ID NO: 12 is used to amplify amplicons from the mutated *N-RAS* allele/template. Such amplicons comprise an Sfu I restriction endonuclease site. Digestion of amplicons generated from such a mutated *N-RAS* allele with Sfu I produces a pattern of nucleic acid fragments, including fragments of 210 and 40 base pairs. Detection of this diagnostic pattern of nucleic acid fragments provides a positive indicator of the presence of the Q61K mutation in the specimen.

In another aspect of the invention, the method is used to detect a *BRCA* allele encoding a mutated *BRCA* polypeptide comprising at least one of the many *BRCA* mutations, including the 185delAG, 5677insA, and 5382insC *BRCA* 1 mutations and the 617delT and 5946delC *BRCA* 2 mutations. In accordance with the method of the invention, each mutation is amplified using specific site-directed mutagenesis primers and standard PCR in 25 µl reactions. Digestion of the amplicon with a specific restriction enzyme such as BsaX I or Bsl I and size fractionation on a 2% agarose gel produces the characteristic bands that are a positive indicator of the presence of the BRCA mutation in the specimen.

In another aspect of the invention, the method is used to detect an *EGFR* allele encoding a mutated *EGFR* polypeptide comprising at least one *EGFR* point mutation. In accordance with the method of the invention, the mutation is amplified using specific site-directed mutagenesis primers and standard PCR. Digestion of the amplicon with a specific restriction enzyme and fractionation on a 2% agarose gel produces the characteristic bands that are a positive indicator of the presence of the mutation in the specimen.

In another aspect of the invention, the method is used to detect a *KRAS* allele encoding a mutated *KRAS* polypeptide comprising at least one *KRAS* point mutation. In

accordance with the method of the invention, the mutated DNA is amplified using specific site-directed mutagenesis primers and standard PCR. Digestion of the amplicon with a specific restriction enzyme and fractionation on a 2% agarose gel produces characteristic bands that are a positive indicator of the presence of the mutation in the specimen.

In another embodiment, the method of the invention is used to detect a mutation associated with resistance to treatment by a virus such as the AIDS virus.

Also presented is a method for detecting a mutation in a target nucleic acid sequence, said method comprising incorporating a restriction enzyme site into a target nucleic acid sequence amplicon, wherein said incorporating is effectuated by polymerase chain reaction (PCR) of a target nucleic acid sequence comprising a mutation, wherein said restriction enzyme site incorporated by PCR comprises said mutation in said target nucleic acid sequence amplicon, and said target nucleic acid sequence amplicon comprising the restriction enzyme site is digestable by said restriction enzyme, and digesting with said restriction enzyme produces a pattern of nucleic acid fragments indicative of a presence of a target nucleic acid sequence comprising a mutation.

The present invention is further directed to a method for diagnosing a disorder associated with a mutation in a target nucleic acid sequence, said method comprising:

(a) providing a plurality of nucleic acid sequences comprising said target nucleic acid sequence, said target nucleic acid sequence comprising said mutation, wherein said mutation does not produce a restriction endonuclease site;

(b) providing a first primer hybridizable near a first portion of said nucleic acid sequence comprising said mutation, said first primer comprising a second portion of said nucleic acid sequence, and a second primer hybridizable to said target nucleic acid sequence, wherein said first and second primer comprise a polymerase chain reaction (PCR) primer pair; wherein one of the primers of this pair changes the DNA sequence of the resultant PCR amplicon to produce a new restriction endonuclease site at the site of the mutation;

(c) performing a PCR amplification of said plurality of nucleic acid sequences using said PCR primer pair, wherein said PCR amplification produces a target nucleic acid sequence amplicon comprising a site recognizable by a restriction endonuclease, said site comprising the first portion and second

portion of said restriction endonuclease site, said first portion and second portion of said restriction endonuclease site operably linked by said PCR amplification;

(d) incubating PCR amplicons produced in step (c) with said restriction endonuclease capable of recognizing said restriction endonuclease site under conditions permissible for said restriction endonuclease activity; and

(e) detecting nucleic acid fragments produced in step (d), wherein detection of a pattern of nucleic acid fragments is indicative of a presence of said target nucleic acid sequence amplicon comprising a site recognizable by said restriction endonuclease,

said pattern of nucleic acid fragments being indicative of a presence of a mutation in said target nucleic acid sequence in said plurality of nucleic acid sequences, wherein said presence of said mutation is a positive diagnostic indicator of a disorder associated with said mutation in said target nucleic acid sequence.

In accordance with the method for diagnosing a disorder associated with a mutation in a target nucleic acid sequence, a target nucleic acid sequence can be an allele associated with a disorder, an oncogene, or a proto-oncogene. Such target nucleic acid sequences are isolated from a biological sample, such as, for example, a patient sample.

In one aspect of the method for diagnosing a disorder associated with a mutation in a target nucleic acid sequence, the presence of a mutation in a target nucleic acid sequence is indicative of the disorder. Such disorders include, but are not limited to genetic diseases, metabolic diseases, and hyperproliferative diseases. An example of a hyperproliferative disease diagnosable using the method of the invention is a cancer.

In another aspect of the method for diagnosing a disorder associated with a mutation in a target nucleic acid sequence, the presence of a mutation in a target nucleic acid sequence is indicative of a predisposition to a disorder. Such disorders include, but are not limited to genetic diseases, metabolic diseases, and hyperproliferative diseases. A predisposition to a hyperproliferative disease such as a cancer, for example, is determinable using the method of the invention.

In one embodiment, the method for diagnosing a disorder associated with a mutation in a target nucleic acid sequence is used to detect a mutation in an oncogene that is an activating point mutation. Such mutations occur in a number of genetic alleles including, but not limited to, a *B-RAF* allele, a *K-RAS* allele, and an *N-RAS* allele. In another embodiment, the method of the invention is used to detect a mutation in an

oncogene that is an inactivating mutation including, but not limited to, a *BRCA* or *EGFR* gene mutation.

In an aspect of the invention, the method for diagnosing a disorder associated with a mutation in a target nucleic acid sequence is used to detect a *B-RAF* allele encoding a mutated *B-RAF* polypeptide comprising a V599E mutation. In accordance with the method of the invention, a PCR primer pair comprising a first primer SEQ ID NO: 7 and a second primer SEQ ID NO: 8 is used to amplify amplicons from the mutated *B-RAF* allele/template. Such amplicons comprise an Alw26 I restriction endonuclease site. Digestion of amplicons generated from a mutated *B-RAF* allele with Alw26 I produces a pattern of nucleic acid fragments, including fragments of 123 and 37 base pairs. Detection of this diagnostic pattern of nucleic acid fragments provides a positive indicator of the presence of the mutation in the specimen.

In another aspect of the invention, the method for diagnosing a disorder associated with a mutation in a target nucleic acid sequence is used to detect an *N-RAS* allele encoding a mutated *N-RAS* polypeptide comprising a Q61R mutation. In accordance with the method of the invention, a PCR primer pair comprising a first primer SEQ ID NO: 9 and a second primer SEQ ID NO: 10 is used to amplify amplicons from the mutated *N-RAS* allele/template. Such amplicons comprise a Bcg I restriction endonuclease site. Digestion of amplicons generated from such a mutated *N-RAS* allele with Bcg I produces a pattern of nucleic acid fragments, including fragments of 168, 34, and 22 base pairs. Detection of this diagnostic pattern of nucleic acid fragments provides a positive indicator of the presence of the mutation in the specimen.

In another aspect of the invention, the method for diagnosing a disorder associated with a mutation in a target nucleic acid sequence is used to detect an *N-RAS* allele encoding a mutated *N-RAS* polypeptide comprising a Q61K mutation. In accordance with the method of the invention, a PCR primer pair comprising a first primer SEQ ID NO: 11 and a second primer SEQ ID NO: 12 is used to amplify amplicons from the mutated *N-RAS* allele/template. Such amplicons comprise an Sfu I restriction endonuclease site. Digestion of amplicons generated from such a mutated *N-RAS* allele with Sfu I produces a pattern of nucleic acid fragments, including fragments of 210 and 40 base pairs. Detection of this diagnostic pattern of nucleic acid fragments provides a positive indicator of the presence of the mutation in the specimen.

In another aspect of the invention, the method for diagnosing a disorder associated with a mutation in a target nucleic acid sequence is used to detect a BRCA allele

encoding a mutated BRCA polypeptide comprising at least one of the many *BRCA* mutations, including the 185delAG, 5677insA, and 5382insC *BRCA* 1 mutations and the 617delT and 5946delC *BRCA* 2 mutations. In accordance with the method of the invention, each mutation is amplified using specific site-directed mutagenesis primers and standard PCR in 25 µl reactions. Digestion of the amplicon with a specific restriction enzyme such as BsaX I or Bsl I and fractionation on a 2% agarose gel produces characteristic bands as a positive indicator of the presence of the mutation in the specimen.

In another aspect of the invention, the method for determining sensitivity to specific treatments associated with a mutation in a target nucleic acid sequence of some types of lung cancer by utilizing point mutations in the epidermal growth factor receptor (EGFR).

In yet another aspect of the invention, the method for diagnosing a disorder associated with a mutation in a target nucleic acid sequence is used to detect the presence or progress of pancreatic cancer by utilizing point mutations in the *K-RAS* gene.

In yet another aspect of the invention the method for diagnosing a disorder associated with a mutation in a target nucleic acid sequence is used to predict resistance to treatment by the AIDS virus.

As described herein, the methods of the present invention are useful for detecting a mutation associated with disease and for diagnosing a disease. Exemplary diseases diagnosable using the methods of the invention include melanoma, breast cancer, lung cancer, pancreatic cancer and AIDS. The present invention also provides a method for indicating disease progression, the clinical stage of the disease, or possible resistance or sensitivity to specific treatments of the disease.

In another embodiment of the invention, there is provided a method for identifying an unknown point mutation by comparison with more than one standard in a single assay, for instance to screen archival tissue samples for the presence of heretofore unsuspected mutations that may have effected treatment outcomes such as unsuspected BRCA mutations in breast cancer patients treated on clinical protocols.

In one aspect of this embodiment, the unknown sample could be run to detect the most common mutations known for a particular type of disease, for example, 45 or more of the most common mutations of the BRCA gene associated with breast cancer.

In another aspect of this embodiment, the unknown sample could be run against the mutations expected to be the most common for a particular genetic heritage.

In another embodiment of the invention, there is provided a fluoroscopic means of determining the results of the restriction reaction.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A, 1B, and 1C show nucleic acid sequences depicting the creation of an Alw26 I restriction site near codon 1796 of *B-RAF* having the V599E mutation, the novel restriction enzyme digestion pattern predicted for a PCR product comprising the novel site, and a photograph of an agarose gel of digested PCR products following electrophoresis, respectively.

Figures 2A, 2B, and 2C show nucleic acid sequences depicting the creation of a Bcg I restriction site near codon 61 of *N-RAS* with a guanine (arrow) replacing an adenine to form the Q61R mutation, the novel restriction enzyme digestion pattern predicted for a PCR product comprising the novel site, and a photograph of an agarose gel of digested PCR products following electrophoresis, respectively.

Figures 3A, 3B, and 3C show nucleic acid sequences depicting the creation of an Sfu I restriction site near codon 61 of *N-RAS* with an adenine (arrow) replacing a cytosine to form the Q61K mutation, the novel restriction enzyme digestion pattern predicted for a PCR product comprising the novel site, and a photograph of an agarose gel of digested PCR products following electrophoresis, respectively.

Figure 4 shows a photograph of an agarose gel following electrophoresis, wherein serially diluted plasmids containing the V599E mutant sequence were analyzed.

Figures 5 A and 5B show immunoblots of cellular extracts probed with antibodies immunologically specific for ERK 1/2, phosphorylated ERK 1/2, or phosphorylated MEK 1/2.

Figure 6 shows a photograph of an agarose gel following electrophoresis wherein enzyme digestion of genomic DNA extracted from paraffin-embedded tumor samples was carried out.

Figures 7A and 7B show nucleic acid sequences depicting the creation of a Bsl I restriction site near codon 1982 of the *BRCA* 2 gene having the 6174delT mutation, and the novel restriction enzyme digestion pattern predicted for a PCR product comprising the novel site.

Figure 8 shows a photograph of an agarose gel of digested PCR products following electrophoresis for 5 different *BRCA* 1 and 2 mutations.

Figure 9A, 9B, 9C and 9D show nucleic acid sequences depicting the creation of a Bcg II restriction site near codon 1796 of *B-RAF* with an adenine (arrow) replacing a thymine to form the V599E mutation, the novel restriction enzyme digestion pattern predicted for a PCR product comprising the novel site, a photograph of an agarose gel of digested PCR products following electrophoresis, and a Southern Blot after site-directed mutagenesis, respectively.

Figure 10 shows a photograph of an agarose gel following electrophoresis of fine needle aspirants of melanoma tumor samples.

DETAILED DESCRIPTION OF THE INVENTION

The method of the present invention is directed to detecting a difference in the sequence of two related nucleic acid sequences. Such differences relate to any mutation including, but not limited to a single base substitution, deletions, or insertions within a sequence that can be specifically recognized by a primer suitable for use in a polymerase chain reaction. The method is applicable to the detection of a known mutation in any biological sample on which PCR may be performed. The method is simple, fast and amenable to automation. It is ideally suited for analysis of patient samples and rapid mutation pre-screening.

A large number of mutations in complex eukaryotic genes comprising a plurality of exons that encode large polypeptides have been shown to be clinically significant. Mutations in such genes have been implicated in a variety of genetic disorders and have been associated with a predisposition for and/or onset of a disease (e.g., cancer). Improved methods for detecting mutations and polymorphisms in such genes are, therefore, needed. In particular, there is a need for methods that can use either DNA or RNA as starting material. Previously described techniques include restriction endonuclease fingerprinting (REF), the single-stranded conformation polymorphism (SSCP) technique, and the protein truncation test (PTT). See above for more detail pertaining to these techniques and impediments to their use. There is also a need for a method that can detect a mutation in a non-coding region of a gene, wherein control elements are frequently located, which could be undetectable using some of the above techniques. Thus, there is a need for improved methods for detecting mutations and polymorphisms in eukaryotic genes, including complex eukaryotic genes. As described herein, the method of the present invention addresses this need.

In one embodiment, the method of the present invention was used successfully to screen a large sample of melanoma samples (N=115) for the presence of the most common B-RAF and N-RAS mutations associated with melanoma. Recent studies have suggested that activating point mutations in B-RAF may commonly occur in melanoma and therefore, detection of such mutations would provide a positive indicator of melanoma. Furthermore, detection of mutations in the B-RAF or N-RAS in melanoma would be useful in stratifying patients on clinical trials testing therapies targeted to the mutations in these genes. Accordingly, as described herein, a method to detect point mutations in heterogeneous tissues containing both wild-type and mutant B-RAF and N-RAS genes was devised which utilizes site-directed mutagenesis to introduce a new restrictions site into a PCR amplicon derived from a template in which a specific point mutation is present (a target nucleic acid sequence). Thus, the presence of a newly incorporated restriction enzyme site into amplicons generated from a mutated template provides a positive indicator for the presence of a mutated template in a heterogeneous population of templates and therefore provides means to detect the mutation.

As presented herein, the method of the invention was used to determine the incidence of mutations in components of mitogen-activated protein kinase (MAPK) pathways in human melanoma. Moreover, corroborative techniques such as immunoblotting (Western blotting) were also used to evaluate and demonstrate downstream up-regulation of MAPK pathways in these tissues.

Of the 115 samples evaluated using the present method, eighty-nine samples (25 of 36 primaries, 18 of 27 regional metastases, 16 of 40 nodal metastases, and 9 of 12 distant metastases) harbored the V599E B-RAF mutation (60 %), seventeen comprised a Q61R N-RAS mutation and four comprised a Q61K N-RAS mutation. Western blotting with anti-phosphorylated ERK 1/2 antibodies demonstrated up-regulation of the MAPK pathway in samples containing activating B-RAF or N-RAS mutations, as compared with MAPK pathway activity in wild-type samples. Of note, there were no false positives detected since the presence of an activating mutation in the MAPK pathway in a sample was confirmed by downstream activation of the pathway in the sample. The method of the invention, therefore, provides a rapid, sensitive, and specific means for detecting a mutation in a biological sample.

As shown herein using the methods of the present invention, activating mutations of the MAPK pathway were present in approximately 60% of samples tested and caused

activation of this cellular pathway. These findings underscore the significance of MAPK pathway activation in the diagnosis and pathogenesis of melanoma.

The method of the present invention for detecting/identifying point mutations, which utilizes a modified site-directed mutagenesis approach, can also be used to detect non-MAPK related mutations. Indeed, any known genetic mutation is potentially detectable using the method of the present invention. The method of the present invention may, for example, be used to advantage for detecting inactivating point mutations in the BRCA genes that are associated with a very high incidence of breast cancer. Multiple point mutations, insertions, and deletions in the BRCA1 and BRCA 2 genes result in the inactivation of these genes. Currently, if a patient with breast cancer is found to harbor a mutation in one of the BRCA genes, family members are routinely screened to determine if they also harbor a mutation in either BRCA 1 or BRCA 2. The presence of such mutations provides a strong positive indicator of a predisposition of an individual for developing breast cancer. The method of the present invention can, therefore, be used to identify individuals at risk for breast cancer, who should be monitored and examined at a greater frequency than patients lacking such mutations. Such individuals may also be treated prophylactically with a variety of prophylactic/therapeutics compounds known to skilled practitioners.

Practicing the method of the present invention for the detection of BRCA mutations is assisted by the availability of the full length sequence of the BRCA genes and characterization of a number of clinically significant mutations in chromosome 13q-linked kindreds. See Tavtigian et al. Nature Genetics, 1996;12(3):333. Screening methods presently employed to detect BRCA mutations involve sequencing of family member's BRCA genes, the nucleic acid sequences of which are generally isolated from a peripheral blood sample. Such methods are expensive and time consuming. Once a mutation has been characterized by sequencing the original patient's BRCA genes, however, the method of the present invention may be used to detect this specific mutation (and/or other BRCA gene mutations) in other family members. Moreover, because inactivating mutations in the BRCA genes are generally germ-line mutations, the family members are anticipated to harbor the same mutation as the index or original patient.

One of ordinary skill in the art could, therefore, in accordance with the method of the present invention, design a specific set of primers or a PCR primer pair capable of incorporating a new diagnostic restriction enzyme site into a PCR amplicon derived from a BRCA gene comprising such a mutation. The presence of the diagnostic restriction

enzyme site in PCR amplicons, as revealed by successful digestion of such amplicons with the specific restriction enzyme would, therefore, be indicative of a presence of the mutated BRCA gene in a patient sample. Thus, the present invention provides a relatively rapid, simple, inexpensive, and accurate method for screening large numbers of family members related to patients diagnosed as having a mutation in a BRCA gene.

There are many uses for a technique such as the method of the invention that detects known specific mutations. Stratifying patients on treatment protocols by the presence or absence of a known genetic mutation such as the V599E *B-RAF* mutation in melanoma is only one example. Another example is when a patient is found to harbor a hereditary mutation such as a BRCA I or II mutation. In this situation the remaining members of the patient's family need to be screened for this specific mutation. The method of the invention can screen these family members quickly and inexpensively. These and other examples of clinically relevant uses of this detection method are all aspects of the invention.

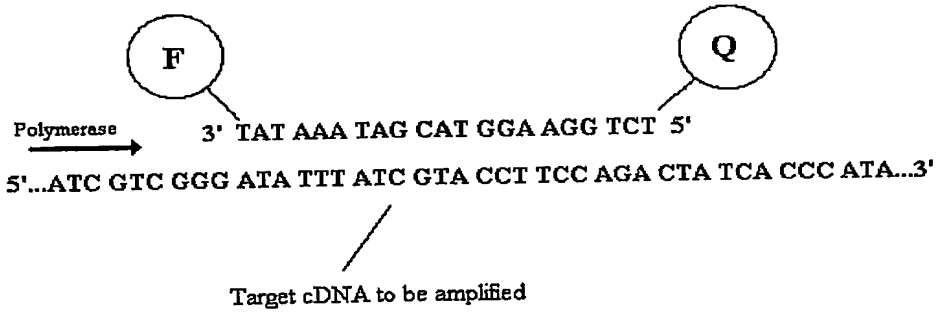
The method of the invention is also useful in other diseases that are associated with point mutations. These include lung cancers associated with mutations of the *EGFR* gene and pancreatic cancers associated with mutations in the *KRAS* gene, as well as other disease such as those associated with the AIDS virus.

The site-directed mutagenesis technique is a very sensitive and specific method for detecting genetic mutations and is inexpensive and easy to use. Furthermore, it is adaptable to any mutation as long as site-directed mutagenesis primers to produce a new restriction site are designed using the mutation as part of the recognition site of the restriction enzyme. While Southern blotting may be necessary to gain maximum sensitivity, it is time consuming and adds to the expense of the method. Furthermore, although ethidum bromide-stained gel electrophoresis or Southern blotting remain easy to perform and are very sensitive and specific methods to detect restriction enzyme digestion of PCR amplicons in this genetic mutation detection method, they limit the throughput of the technique. Therefore it is advantageous to develop a high throughput modification of the basic site-directed mutagenesis detection technique if it is to become a more useful method of detecting genetic mutations in the clinical setting. A technique to increase throughput and maintain sensitivity and specificity is fluorescent detection of the restriction enzyme digestion.

Accordingly, one embodiment of this invention relates to a high throughput genetic mutation detection method based on the site-directed mutagenesis detection

technique. The requirements for this high throughput method are: 1) Specificity and sensitivity equivalent to the basic site-directed mutagenesis method and direct sequencing. 2) Adaptability to an automated system format. 3) Rapid, inexpensive and easy to use compared to existing systems such as direct sequencing. To meet these requirements a fluorescence-based detection system is used. Fluorescence-based detection systems such as quantitative real-time polymerase chain reaction (QRT-PCR) systems can detect very minute differences in fluorescence making them very sensitive. See Bustin, J. Clin Endocrinol Metab. Sep 2003:29(1):23-39, Heid et al, Genome Res. 1996;(10):986-994. Combined with the specificity of restriction enzyme digestion, a fluorescence-based detection method yields an acceptable high-throughput system. Two different fluorescence-based detection methods that have the potential to meet the requirements listed above can be described as follows.

The first approach is similar to the fluorophore-quencher method used in Taqman QRT-PCR techniques (Bustin, Heid). Taqman probes incorporate a fluorophore that emits energy at a particular wavelength that can be detected by the QRT-PCR device. The probe also incorporates a quencher molecule that absorbs the fluorescence from the fluorophore when the quencher is within 10 to 100 angstroms of the fluorophore. The site-directed mutagenesis method of genetic mutation detection uses specially designed primers that insert changes in the amplicon of a PCR reaction that result in the formation of a new restriction site only when the mutation of interest is present. The digestion of the amplicon with the specific restriction enzyme results in cutting of the amplicon into two or more discrete oligonucleotides. The typical design of a Taqman probe for QRT-PCR is shown schematically as

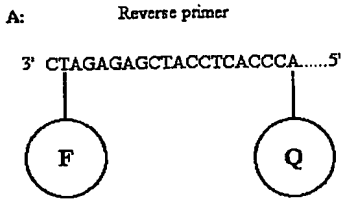


As the polymerase elongates the complementary strand, the bound probe is lysed by Taq Polymerase's native 5' endonuclease activity, which releases the fluorophore (F) from the probe and as the fluorophore leaves the vicinity of the quencher (Q) fluorescence is produced. This fluorescence is detected by the QRT-PCR devise and the number of PCR cycles that it takes to reach a predetermined threshold of fluorescence is proportional to the amount of starting cDNA in the sample (Bustin).

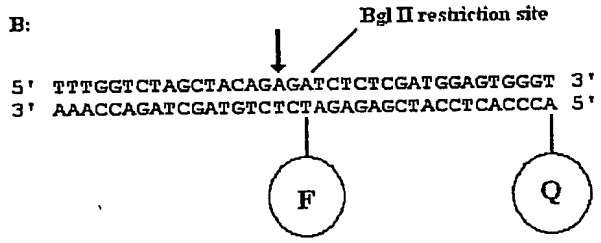
In order to adapt this system to fluorescence-based detection the fluorophore and the quencher are placed on different sides of the restriction site produced in the mutant amplicon. When the amplicon is intact, the fluorophore and the quencher are in close proximity and no fluorescence is emitted. If the amplicon contains the mutation the restriction site will be present and the amplicon will be cleaved by the specific restriction enzyme. Because the fluorophore and the quencher are on different sides of the restriction site the fluorophore leaves the vicinity of the quencher and fluorescence is produced. The increase in fluorescence is detected on enzymatic digestion of the amplicon using a QRT-PCR devise or a simple fluorometer. Both devices are sensitive enough to detect very small changes in fluorescence over background and very small numbers of mutant amplicons can be detected in a WT background.

The next step is to place the fluorophore and the quencher on opposite sides of the restriction site of the amplicon. Two potential techniques for producing a double-labeled amplicon with a fluorophore on one side of the restriction site and a quencher on the other are as follows:

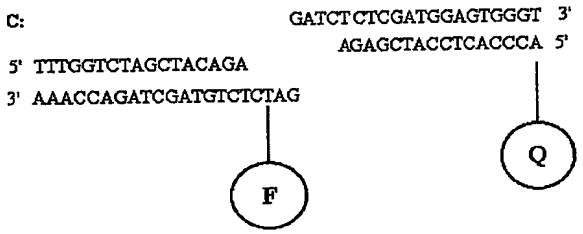
1: The Dual-labeled Primer Method. This method is based on the fact that restriction enzymes often do not cut at the recognition site of the enzyme but upstream or downstream of this site. A primer is designed that places the fluorophore on the far site of the resultant restriction site. In this method a reverse primer designed so that a fluorophore is attached to the thymine one base from the 3' end and a quencher is attached at the 5' end. This produces a double labeled oligonucleotide less then 100 angstroms long so the quencher absorbs the fluorophore's fluorescence as shown in A.



When used in a PCR reaction this reverse primer incorporates the fluorophore and the quencher into the antisense strand of the double stranded product. The reverse primer produces a Bgl II restriction site using site-directed mutagenesis. The arrow indicates the adenine that replaces the thymine to produce the V599E mutation as shown in B.



When the amplicon is cleaved with the restriction enzyme Bgl II a four base overhang is produced. This leaves the fluorophore on one fragment and the quencher on the other as shown in C and fluorescence is produced.

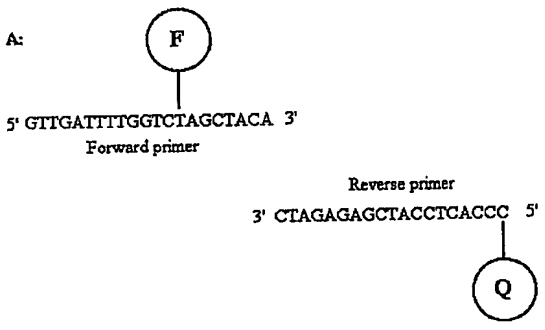


Placing the fluorophore and the quencher on the same primer has the advantage of allowing long amplicons because the other primer is designed to bind as far away from the dual labeled primer as necessary. This allows the production of digestion products large enough to be easily detected by standard ethidium-bromide stained gel electrophoresis, and that in turn allows the determination of the proper conditions for optimal PCR and restriction enzyme digestion without the need for fluorescent detection of the products. A second advantage is that there is no unquenched fluorophore in the reaction mix, minimizing background fluorescence that will likely improve the assay's

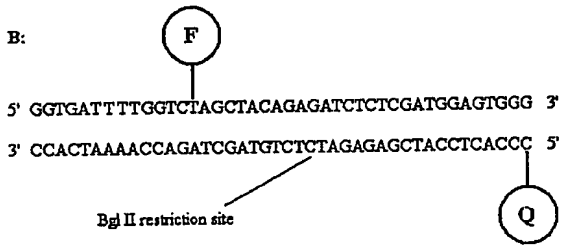
sensitivity. One example of this type of enzyme is Bcg-1 used to detect the Q61R *N-RAS* mutation (Figure 2). Bcg-1 cuts the amplicon 10 to 12bp downstream from the recognition site and the fluorophore can be placed in many different locations along the reverse primer, keeping it away from both the 3' end and the Bcg-1 recognition region. However, some enzymes leave little or no overlap on digestion. One such enzyme is Sfu-1 used to detect the Q61K *N-RAS* mutation (Figure 3). Sfu-1 digestion results in only a 2bp overhang, leaving few options for fluorophore placement. Amplicons that must be cut with an enzyme such as Sfu-1 generally require a different strategy.

The second method to place a fluorophore and a quencher on different sides of a restriction site in the site-directed mutagenesis amplicon uses different labeled primers The fluorophore is placed on the forward primer and the quencher is placed on the reverse primer and a standard PCR reaction is performed. A much shorter amplicon is required to keep the fluorophore and the quencher in close enough proximity for fluorescent quenching to occur.

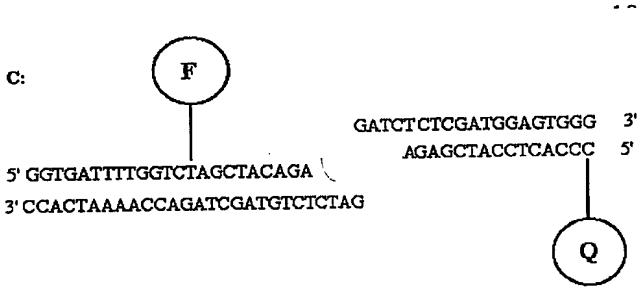
In particular, primers designed to produce a new Bgl II site in V599E mutant *B-RAF* (site-directed mutagenesis primers) are shown in A, below. A quencher is attached to the 5' end of the reverse primer and a fluorophore is attached to the 14th base (thymine) of the forward primer. The fluorophore is not attached to the 5' end of the forward primer because this would result in a greater then 100 angstrom separation between the quencher and the fluorophore in the resultant amplicon.



The amplicon produced with these primers is shown in B. The arrow indicates the adenine that replaces the thymine to produce the V599E mutation.



When the amplicon is digested with Bgl II the fluorophore and the quencher are separated and fluorescence is produced as shown in C.

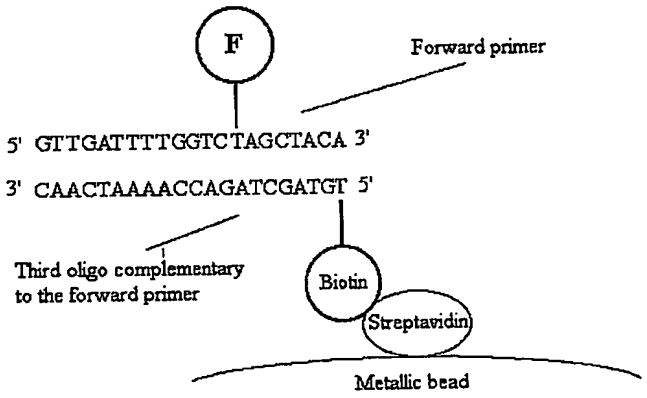


The two-labeled primers method avoids the possible problems of the fluorophore interfering with Taq polymerase function or restriction enzyme cleavage by moving the fluorophore far back from the 3' end of the primer. An advantage of this method is that it can be adapted to almost any set of primers produced to detect any genetic mutation with almost any restriction enzyme.

A disadvantage to this method is the presence of a large excess of unquenched fluorophore in the reaction mix in the form of unused primer. Because PCR reactions require an excess of both forward and reverse primers to gain maximal efficiency (leading to maximal detection sensitivity) it can be difficult to lower the amount of fluorophore-bound primer in the reaction mix without loosing sensitivity of detection. Removing the

excess primer through standard PCR cleanup techniques can be difficult, if not impossible, because the amplicon is not much more than twice the size of the primers and most methods to remove excess primers and template cDNA from PCR reactions rely on the usual large size differential between the amplicon and the primers. Therefore, a method to remove excess fluorophore-labeled primer from the reaction has been designed.

One way to remove the fluorophore-bound primers without loosing significant amounts of the amplicon is to design a third oligonucleotide complimentary to the fluorophore-bound primer. This third oligonucleotide is designed with a biotin molecule bound to the 5' end.

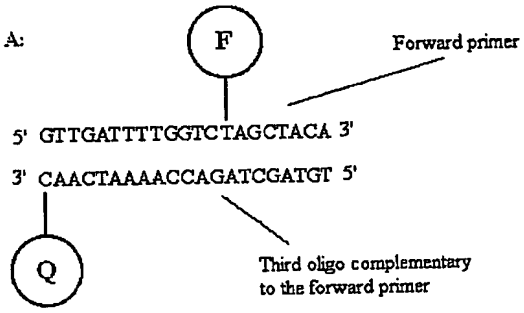


The PCR reaction is run using the forward and reverse primers. Once the PCR reaction is completed the biotin-bound oligonucleotide is added to the PCR reaction mix and incubated for a short time. The PCR mix is then mixed with magnetic beads that are coated with streptavidin. The biotin binds to the streptavidin and removes the excess fluorophore-bound forward primer from the PCR mix. A magnetic stand is used to remove the metallic beads from solution and the PCR mix is removed. Restriction enzyme digestion and fluorescent detection are then carried out using the PCR mix.

An important consideration in this respect is the melting temperatures of the forward primer/third oligo and the amplicon. If a PCR amplicon is produced with a melting temperature much higher than the melting temperature of the forward primer/third olig, binding of the third primer to the amplicon is minimized. Because the

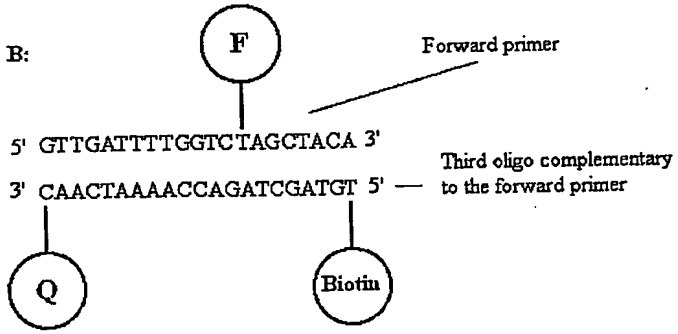
amplicon is over twice the length of the forward primer/oligo, engineering a higher melting temperature in the amplicon should not be a problem.

Another method for suppressing the background fluorescence produced by excess fluorophore-bound forward primer is to quench the excess primer. To do this a different third oligonucleotide that is complementary to the fluorophore-bound forward primer is produced but instead of a biotin molecule a quencher is added to the 3' end of this third oligo. After the PCR reaction an excess of the quencher-bound third oligo is added to the reaction mix and incubated for a short time. This third oligo binds to the excess fluorophore-bound primer and quenches the fluorescence.



It is also possible to combine the biotin and quencher methods, adding first the biotin third oligo, extracting the excess primer, and then adding the quenching oligo to quench any remaining background.

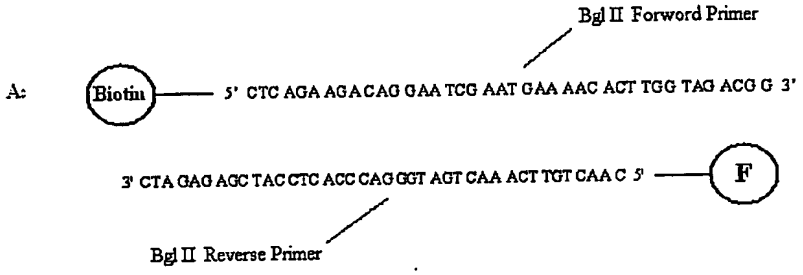
Alternatively one oligo that combines the quencher and biotin molecules is produced. This combined oligo extracts and quenches in one step.



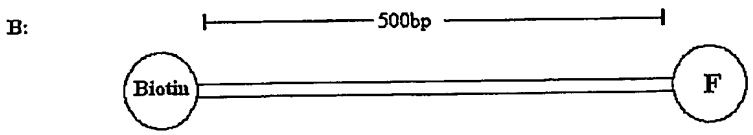
After addition of this dual labeled oligo a solution containing streptavidin-bound metallic beads is added to the PCR mixture. The biotin will bind to the streptavidin and

remove the excess fluorophore-bound forward primer from the PCR mix. A magnetic stand is used to remove the metallic beads from solution and the PCR mix is removed. Any excess fluorophore-bound forward primer not removed by the biotin/strepavidin step will be quenched. Restriction enzyme digestion and fluorescent detection are then carried out using the PCR mix.

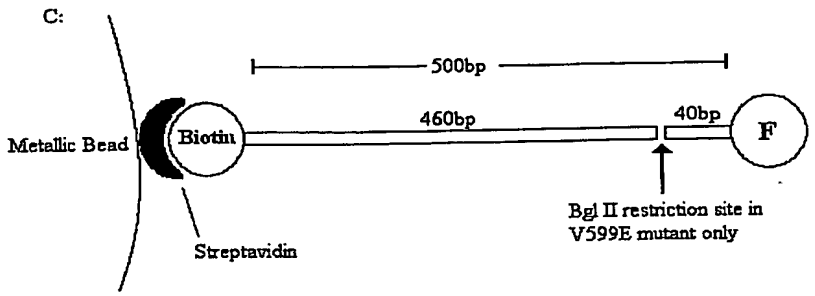
A second method to detect restriction enzyme digestion of the mutant amplicon uses the binding of biotin to strepavidin to remove WT amplicon from the PCR mixture. A reverse primer with a fluorophore bound to the 5' end and a forward primer with a biotin bound to the 5' end are developed. The primers produce a 500bp amplicon and incorporate the site-directed mutagenesis system to insert a new restriction site in mutant amplicons. For example, a reverse primer uses site-directed mutagenesis to produce a new Bgl II restriction site if the V599E mutation is present. A fluorophore (F) is bound to the 5' end of the reverse primer and a biotin molecule is bound to the 5' end of the forward primer.



An amplicon is produced as shown in B:



The amplicon is bound by the biotin molecule to a streptavidin-coated metallic bead. The amplicon is cut by Bgl II if the V599E mutation is present, releasing the fluorophore into the supernatant.



Standard PCR is performed using the fluorophore-bound forward and biotin-bound reverse primers to produce a long amplicon (500bp or more). The PCR product is washed using a standard spin-column cleanup system (Promega) to remove excess primers. The washed PCR mixture is added to metallic beads coated with streptavidin (MagneSphere, Promega, Madison, WI). The Biotin molecule binds to the beads in solution and a magnetic stand provided by the manufacturer of the streptavidin-coated beads (Promega) is used to remove the bead/amplicon complex from solution. The supernatant is removed and the bead/amplicon complex washed to remove any residual template and primers. Restriction enzyme and buffer are then added and the bead/amplicon complex is returned to solution. Restriction digestion is carried out for two hours followed by removal of the bead/amplicon complexes from solution with the magnetic stand. If the mutation of interest is present in the amplicons the fluorophore is released into the supernatant by enzyme digestion. A fluorometer is used to measuring the fluorescence of the supernatant to detect restriction enzyme cutting.

Advantages of the fluorophore/biotin system include the lack of a need for a quencher molecule; long amplicons can be produced with the fluorophore far from the restriction enzyme recognition site and the site of restriction enzyme cutting. Second, this system can be adapted to almost any restriction enzyme-primer pair, making development of detection systems for other mutations easier. Third, long amplicons allow the use of standard PCR cleanup methods for the removal of excess primers to lower background fluorescence. Finally, using metallic beads to remove the amplicon from solution allows restriction enzyme digestion in the proper buffer without the addition of contaminating PCR buffer from the PCR reaction.

An ordinarily skilled practitioner could readily apply the method of the present invention to a characterized mutation in any allele or gene. Indeed, all that is required to practice the present method is knowledge pertaining to the nucleic acid sequence of a mutated site and the nucleic acid sequences flanking the mutation in question. With such information and in accordance with the invention, an ordinarily skilled artisan could readily design a specific PCR primer pair to incorporate a diagnostic restriction enzyme site into a PCR amplicon generated from a template comprising a mutation in question, perform a PCR amplification to produce PCR amplicons, and analyze the resultant nucleic acid fragments produced to determine if a template comprising the mutation is present in a sample.

In order to more clearly set forth the parameters of the present invention, the following definitions are used:

As used in the specification and appended claims, the singular forms "a", "an", and "the" include plural references unless the context clearly dictates otherwise. Thus for example, reference to "the method" includes one or more methods, and/or steps of the type described herein and/or which will become apparent to those persons skilled in the art upon reading this disclosure and so forth.

The phrase "flanking nucleic acid sequences" refers to those contiguous nucleic acid sequences that are 5' and 3' to a particular nucleic acid (e.g., a point mutation) or stretch of nucleic acid sequences (e.g., an endonuclease cleavage site).

The term "restriction endonuclease" refers to an enzyme that can cleave DNA internally.

The term "complementary" refers to two DNA strands that exhibit substantial normal base pairing characteristics. Complementary DNA may, however, contain one or more mismatches.

The term "hybridization" refers to the hydrogen bonding that occurs between two complementary DNA strands.

The term "specifically hybridize" refers to the association between two single-stranded nucleic acid molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially complementary"). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded DNA or RNA molecule of the invention, to the substantial exclusion of

hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence.

The terms "percent similarity", "percent identity" and "percent homology" when referring to a particular sequence are used as set forth in the University of Wisconsin GCG software program and are known in the art.

As used herein, a "nucleic acid" is a compound or composition that is a polymeric nucleotide or polynucleotide. Nucleic acids include both nucleic acids and fragments thereof from any source, in purified or unpurified form, including DNA (dsDNA and ssDNA) and RNA (e.g., t-RNA, m-RNA, r-RNA), mitochondrial DNA and RNA, chloroplast DNA and RNA, DNA-RNA hybrids, or mixtures thereof, genes, chromosomes, plasmids, the genomes of biological material such as microorganisms, e.g., bacteria, yeasts, viruses, viroids, molds, fungi, plants, animals, and humans. Sequences of interest may be embedded in sequences of any length of, for example, a chromosome, cDNA, or plasmid. The nucleic acid may be only a minor fraction of a complex mixture such as that of a biological sample. The nucleic acid may be obtained from a biological sample by procedures well known in the art.

With reference to nucleic acids of the invention, the term "isolated nucleic acid" is sometimes used. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous in the naturally occurring genome of the organism in which it originated. For example, an "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a prokaryotic or eukaryotic cell or host organism.

In applications wherein the nucleic acid is RNA, it may first be converted to cDNA by means of a primer and reverse transcriptase. The nucleotide polymerase used in the present invention for carrying out amplification and chain extension can have reverse transcriptase activity. A nucleic acid sequence may comprise a particular gene or mutant thereof of biological interest such as, for example, the hemoglobin gene associated with sickle-cell anemia, the cystic fibrosis gene, and a number of oncogenes and proto-oncogenes.

When applied to RNA, the term "isolated nucleic acid" refers primarily to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from other nucleic acids with which it is generally associated in its natural state (i.e., in cells or tissues). An

isolated nucleic acid (either DNA or RNA) may further represent a molecule produced directly by biological or synthetic means and separated from other components present during its production.

As used herein, a "sample" refers to a material suspected of containing a nucleic acid. Such samples include, but are not limited to biological fluids such as blood, serum, plasma, sputum, lymphatic fluid, semen, vaginal mucus, feces, urine, spinal fluid and amniotic fluid; and biological tissue such as a tumor sample (excised or aspirated, e.g.,) or hair and skin. Other samples include cell cultures and the like, plants, food, forensic samples such as paper, fabrics and scrapings, water, sewage, and medicinals. When necessary, the sample may be pretreated with reagents to liquefy the sample and release the nucleic acids from binding substances. Such pretreatments are well known in the art.

As used herein, "amplification of nucleic acids" refers to any method that results in the formation of one or more copies of a nucleic acid (exponential amplification). One such method for enzymatic amplification of specific sequences of DNA is known as the polymerase chain reaction (PCR), as described by Saiki et al. (1986) Science, 230: 1350-1354. This *in vitro* amplification procedure is based on repeated cycles of denaturation, oligonucleotide primer annealing, and primer extension by a thermophilic template dependent polynucleotide polymerase, resulting in an exponential increase in copies of the desired sequence of the nucleic acid flanked by the primers. The two different PCR primers are designed to anneal to opposite strands of the DNA at positions that allow the polymerase catalyzed extension product of one primer to serve as a template strand for the other, leading to the accumulation of a discrete double stranded fragment whose length is defined by the distance between the 5' ends of the oligonucleotide primers. Primer length can vary from about 10 to 50 or more nucleotides and primers are usually selected to be at least about 15 nucleotides to ensure high specificity. The double stranded fragment that is produced is called an "amplicon" and may vary in length form as few as about 30 nucleotides to 10,000 or more.

As used herein, "chain extension of nucleic acids" refers to extension of the 3'-end of a polynucleotide in which additional nucleotides or bases are appended. Chain extension relevant to the present invention is template dependent, that is, the appended nucleotides are determined by the sequence of a template nucleic acid to which the extending chain is hybridized. The chain extension product sequence that is produced is complementary to the template sequence. Usually, chain extension is enzyme catalyzed, preferably, in the present invention, by a thermophilic DNA polymerase.

As used herein, "target nucleic acid sequence" refers to a sequence of nucleotides to be studied either for the presence of a difference from a related sequence or for the determination of its presence or absence. The target nucleic acid sequence may be double stranded or single stranded and from a natural or synthetic source. When the target nucleic acid sequence is single stranded, the method of the present invention produces a nucleic acid duplex comprising the single stranded target nucleic acid sequence.

The target sequence usually exists within a portion or all of a nucleic acid, the identity of which is known to an extent sufficient to allow preparation of various primers necessary for introducing a restriction enzyme site into or near a target sequence or conducting an amplification of the target sequence or a chain extension of the products of such an amplification in accordance with the present invention. Accordingly, other than for the sites to which the primers bind, the identity of the target nucleic acid sequence may or may not be known. In general, in PCR, primers hybridize to, and are extended along (chain extended), a target sequence, and, thus, the target sequence acts as a template. The target sequence usually contains from about 30 to 20,000 or more nucleotides, more frequently, 100 to 10,000 nucleotides, preferably, 50 to 1,000 nucleotides. The target nucleic acid sequence is generally a fraction of a larger molecule or it may be substantially the entire molecule. The minimum number of nucleotides in the target sequence is selected to assure that a determination of a difference between two related nucleic acid sequences in accordance with the present invention can be achieved.

As used herein, a "reference nucleic acid sequence" refers to a nucleic acid sequence that is related to the target nucleic acid in that the two sequences are identical except for the presence of a difference, such as a mutation. Where a mutation is to be detected, the reference nucleic acid sequence usually contains the normal or "wild type" sequence. In certain situations the reference nucleic acid sequence may be part of a sample as, for example, in samples from tumors, the identification of partially mutated microorganisms, or identification of heterozygous carriers of a mutation. Consequently, both the reference and the target nucleic acid sequences are subjected to similar or the same amplification conditions. As with the target nucleic acid sequence, the identity of the reference nucleic acid sequence need be known only to an extent sufficient to allow preparation of various primers necessary for conducting an amplification of the reference sequence or a chain extension of the products of such amplification in accordance with the present invention. Accordingly, other than for the sites to which the primers bind, the identity of the reference nucleic acid sequence may or may not be known.

The reference nucleic acid sequence may be a reagent employed in the methods of the present invention. Reference nucleic acid sequences are particularly useful in PCR amplification for detection of a target nucleic acid sequence. The reference nucleic acid reagent may be obtained from a natural source or prepared by known methods such as those described below in the definition of oligonucleotides.

As used herein, the phrase "related nucleic acid sequences" refers to two nucleic acid sequences that contain at least 15 nucleotides at each end that are identical but have different lengths or have intervening sequences that differ by at least one nucleotide. Frequently, related nucleic acid sequences differ from each other by a single nucleotide. Such a difference is referred to herein as the "difference between two related nucleic acid sequences." A difference can be produced by the substitution, deletion or insertion of any single nucleotide or a series of nucleotides within a sequence.

As used herein, a "mutation" refers to a change in the sequence of nucleotides of a normally conserved nucleic acid sequence resulting in the formation of a mutant as differentiated from the normal (unaltered) or wild type sequence. Mutations tend to be categorized into two general classes: base-pair substitutions and frameshift mutations. The latter entail the insertion or deletion of one to several nucleotide pairs. A difference of one nucleotide can be significant as to phenotypic normality or abnormality as in the case of, for example, sickle cell anemia.

As used herein, a "duplex" is a double stranded nucleic acid sequence wherein a majority of the nucleotides therein are complementary.

The term "oligonucleotide," as used herein refers to primers and probes of the present invention, and is defined as a nucleic acid molecule comprised of two or more ribo- or deoxyribonucleotides, preferably more than three. The exact size of the oligonucleotide will depend on various factors and on the particular application for which the oligonucleotide is used.

Various techniques can be employed for preparing an oligonucleotide utilized in the present invention. Such oligonucleotides can be obtained by biological synthesis or by chemical synthesis. For short sequences (up to about 100 nucleotides), chemical synthesis will frequently be more economical as compared to biological synthesis. In addition to economy, chemical synthesis provides a convenient way of incorporating low molecular weight compounds and/or modified bases during the synthesis step. Furthermore, chemical synthesis is flexible with regard to the length and region of the target polynucleotide binding sequence. An oligonucleotide may be synthesized by standard

methods such as those used in commercial automated nucleic acid synthesizers. Chemical synthesis of DNA on a suitably modified glass or resin can result in DNA covalently attached to the surface. This may offer advantages in washing and sample handling. For longer sequences, standard replication methods employed in molecular biology can be used such as the use of M13 for single stranded DNA as described by Messing (1983) Methods Enzymol, 101, 20-78.

Other methods of oligonucleotide synthesis include phosphotriester and phosphodiester methods (Narang et al. (1979) Meth. Enzymol 68: 90) and synthesis on a support (Beaucage et al. (1981) Tetrahedron Letters 22: 1859-1862) as well as the phosphoramidate technique, Caruthers et al., "Methods in Enzymology," Vol. 154, pp. 287-314 (1988), and others described in "Synthesis and Applications of DNA and RNA," Narang, editor, Academic Press, New York, 1987, and the references contained therein.

The term "primer" as used herein refers to an oligonucleotide, either RNA or DNA, either single-stranded or double-stranded, either derived from a biological system, generated by restriction enzyme digestion, or produced synthetically which, when placed in the proper environment, is able to act functionally as an initiator of template-dependent nucleic acid synthesis. When presented with an appropriate nucleic acid template, suitable nucleoside triphosphate precursors of nucleic acids, a polymerase enzyme, suitable cofactors and conditions such as a suitable temperature and pH, the primer may be extended at its 3' terminus by the addition of nucleotides by the action of a polymerase or similar activity to yield a primer extension product. The primer may vary in length depending on the particular conditions and requirement of the application. For example, in diagnostic applications, the oligonucleotide primer is typically 15-25 or more nucleotides in length. The primer must be of sufficient complementarity to the desired template to prime the synthesis of the desired extension product, that is, to be able anneal with the desired template strand in a manner sufficient to provide the 3' hydroxyl moiety of the primer in appropriate juxtaposition for use in the initiation of synthesis by a polymerase or similar enzyme. It is not required that the primer sequence represent an exact complement of the desired template. For example, a non-complementary nucleotide sequence may be attached to the 5' end of an otherwise complementary primer. Alternatively, non-complementary bases may be interspersed within the oligonucleotide primer sequence, provided that the primer sequence has sufficient complementarity with the sequence of the desired template strand to functionally provide a template-primer complex for the synthesis of the extension product.

For applications wherein a primer is employed in a chain extension on a polynucleotide template such as in, for example, an amplification of a nucleic acid, the primer is usually a synthetic oligonucleotide that is single stranded and comprises a hybridizable sequence at its 3'-end that is capable of hybridizing with a defined sequence of the target or reference polynucleotide (nucleic acid sequence). In general, a hybridizable sequence of such a primer has at least 90%, preferably 95%, most preferably 100%, complementarity to a defined sequence or primer binding site. The number of nucleotides in the hybridizable sequence of an oligonucleotide primer should be such that stringency conditions used to hybridize the oligonucleotide primer will prevent excessive random non-specific hybridization. Usually, the number of nucleotides in the hybridizable sequence of the oligonucleotide primer will be at least ten nucleotides, preferably at least 15 nucleotides and, preferably 20 to 50, nucleotides.

The term "probe" as used herein refers to an oligonucleotide, polynucleotide or nucleic acid, either RNA or DNA, whether occurring naturally as in a purified restriction enzyme digest or produced synthetically, which is capable of annealing with or specifically hybridizing to a nucleic acid with sequences complementary to the probe. A probe may be either single-stranded or double-stranded. The exact length of the probe will depend upon many factors, including temperature, source of probe and use of the method. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide probe typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides. The probes herein are selected to be "substantially" complementary to different strands of a particular target nucleic acid sequence. This means that the probes must be sufficiently complementary so as to be able to "specifically hybridize" or anneal with their respective target strands under a set of pre-determined conditions. Therefore, the probe sequence need not reflect the exact complementary sequence of the target. For example, a non-complementary nucleotide fragment may be attached to the 5' or 3' end of the probe, with the remainder of the probe sequence being complementary to the target strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the probe, provided that the probe sequence has sufficient complementarity with the sequence of the target nucleic acid to anneal therewith specifically.

The term "nucleoside triphosphates" refers to nucleosides having a 5'-triphosphate substituent. The nucleosides are pentose sugar derivatives of nitrogenous bases of either purine or pyrimidine derivation, covalently bonded to the 1'-carbon of the pentose sugar,

which is usually a deoxyribose or a ribose. The purine bases comprise adenine(A), guanine (G), inosine (I), and derivatives and analogs thereof. The pyrimidine bases comprise cytosine (C), thymine (T), uracil (U), and derivatives and analogs thereof. Nucleoside triphosphates include deoxyribonucleoside triphosphates such as the four common triphosphates dATP, dCTP, dGTP and dTTP and ribonucleoside triphosphates such as the four common triphosphates rATP, rCTP, rGTP and rUTP.

The term "nucleoside triphosphates" also includes derivatives and analogs thereof, which are exemplified by those derivatives that are recognized and polymerized in a similar manner to the underivatized nucleoside triphosphates. Examples of such derivatives or analogs, by way of illustration and not limitation, are those which are biotinylated, amine modified, alkylated, and the like and also include phosphorothioate, phosphite, ring atom modified derivatives, and the like.

The term "nucleotide" refers to a base-sugar-phosphate combination that is the monomeric unit of nucleic acid polymers, i.e., DNA and RNA.

The term "modified nucleotide" refers to a unit in a nucleic acid polymer that results from the incorporation of a modified nucleoside triphosphate during an amplification reaction and therefore becomes part of the nucleic acid polymer.

The term "nucleoside" refers to a base-sugar combination or a nucleotide lacking a phosphate moiety.

A "nucleotide polymerase" is a catalyst, usually an enzyme, for forming an extension of a polynucleotide along a DNA or RNA template wherein the extension is complementary thereto. The nucleotide polymerase is a template dependent polynucleotide polymerase and utilizes nucleoside triphosphates as building blocks for extending the 3'-end of a polynucleotide to provide a sequence complementary with the polynucleotide template. Usually, the catalysts are enzymes, such as DNA polymerases, for example, prokaryotic DNA polymerase (I, II, or III), T4 DNA polymerase, T7 DNA polymerase, Klenow fragment, and reverse transcriptase, and are preferably thermally stable DNA polymerases such as, for example, Vent® DNA polymerase, VentR® DNA polymerase, Pfu® DNA polymerase, and Taq® DNA polymerase, derived from any source such as cells, bacteria, such as *E. coli*, plants, animals, virus, and thermophilic bacteria.

The phrase "wholly or partially sequentially" refers to applications wherein the sample and various agents utilized in the present invention are combined other than concomitantly (simultaneously), one or more may be combined with one or more of the

remaining agents to form a subcombination. Subcombination and remaining agents can then be combined and can be subjected to the present method.

When used in the context of nucleotide sequences, "hybridization (hybridizing) or binding"are used interchangeably herein. The ability of two nucleotide sequences to hybridize to each other is based on the degree of complementarity of the nucleotide sequences, which in turn is based on the fraction of matched complementary nucleotide pairs. The degree of complementarity between two sequences is a significant factor to be considered with regard to the stringency of hybridization conditions utilizable and the specificity of the binding anticipated for the two sequences. Increased stringency is achieved by elevating the temperature, increasing the ratio of cosolvents, lowering the salt concentration, and the like.

"Complementary" refers to two sequences which can bind to each other in an anti-parallel sense wherein the 3'-end of each sequence binds to the 5'-end of the other sequence and each A, T(U), G, and C of one sequence is then aligned with a T(U), A, C, and G, respectively, of the other sequence.

A "copy" may, for example, refer to a sequence that is a direct identical copy of a single stranded polynucleotide sequence as differentiated from a sequence that is complementary to the sequence of such a single stranded polynucleotide.

As used herein, "conditions for extending a primer" includes a nucleotide polymerase, nucleoside triphosphates or analogs thereof capable of acting as substrates for the polymerase and other materials and conditions required for enzyme activity such as a divalent metal ion (usually magnesium), pH, ionic strength, organic solvent (such as formamide), and the like.

A member of a specific binding pair ("sbp member") refers to one of two different molecules, having an area on the surface or in a cavity which specifically binds to and is thereby defined as complementary with a particular spatial and polar organization of another molecule. The members of the specific binding pair are referred to as ligand and receptor (antiligand). These may be members of an immunological pair such as antigen-antibody, or may be operator-repressor, nuclease-nucleotide, biotin-avidin, hormone-hormone receptor, IgG-protein A, DNA--DNA, DNA-RNA, and the like.

As used herein, "ligand" refers to any compound for which a receptor naturally exists or can be prepared.

As used herein, a "receptor "("antiligand") refers to any compound or composition capable of recognizing a particular spatial and/or polar organization of a molecule, e.g.,

epitope or determinant site. Illustrative receptors include naturally occurring and synthetic receptors, e.g., thyroxine binding globulin, antibodies, enzymes, Fab fragments, lectins, nucleic acids, repressors, oligonucleotides, protein A, complement component C1q, or DNA binding proteins and the like.

As used herein, a "small organic molecule" refers to a compound of molecular weight less than about 1500, preferably 100 to 1000, more preferably 300 to 600 such as biotin, digoxigenin, fluorescein, rhodamine and other dyes, tetracycline and other protein binding molecules, and haptens, etc. A small organic molecule can provide a means for attachment of a nucleotide sequence to a label or to a support.

As used herein, a "support or surface" refers to a porous or non-porous water insoluble material. The support can be hydrophilic or capable of being rendered hydrophilic and includes inorganic powders such as silica, magnesium sulfate, and alumina; natural polymeric materials, particularly cellulosic materials and materials derived from cellulose, such as fiber containing papers, e.g., filter paper, chromatographic paper, etc.; synthetic or modified naturally occurring polymers, such as nitrocellulose, cellulose acetate, poly (vinyl chloride), polyacrylamide, cross linked dextran, agarose, polyacrylate, polyethylene, polypropylene, poly(4-methylbutene), polystyrene, polymethacrylate, poly(ethylene terephthalate), nylon, poly(vinyl butyrate), etc.; either used by themselves or in conjunction with other materials; glass available as Bioglass, ceramics, metals, and the like. Natural or synthetic assemblies such as liposomes, phospholipid vesicles, and cells can also be employed.

Binding of sbp members to a support or surface may be accomplished by well-known techniques, commonly available in the literature. See, for example, "Immobilized Enzymes," Chibata, Halsted Press, New York (1978) and Cuatrecasas, J. Biol. Chem., 245:3059 (1970). The surface can have any one of a number of shapes, such as strip, rod, particle, including bead, and the like.

As used herein, a "label" refers to a member of a signal producing system. Labels include reporter molecules that can be detected directly by virtue of generating a signal, and specific binding pair members that may be detected indirectly by subsequent binding to a cognate that contains a reporter molecule such as oligonucleotide sequences that can serve to bind a complementary sequence or a specific DNA binding protein; organic molecules such as biotin or digoxigenin that can bind respectively to streptavidin and antidigoxin antibodies, respectively; polypeptides; polysaccharides; and the like. In general, any reporter molecule that is detectable can be used. The reporter molecule can

be isotopic or nonisotopic, usually non-isotopic, and can be a catalyst, such as an enzyme, dye, fluorescent molecule, chemiluminescer, coenzyme, enzyme substrate, radioactive group, a particle such as latex or carbon particle, metal sol, crystallite, liposome, cell, etc., which may or may not be further labeled with a dye, catalyst or other detectable group, and the like. The reporter group can be a fluorescent group such as fluorescein, a chemiluminescent group such as luminol, a terbium chelator such as N-(hydroxyethyl) ethylenediaminetriacetic acid that is capable of detection by delayed fluorescence, and the like.

The label is a member of a signal producing system and can generate a detectable signal either alone or together with other members of the signal producing system. As mentioned above, a reporter molecule can serve as a label and can be bound directly to a nucleotide sequence. Alternatively, the reporter molecule can bind to a nucleotide sequence by being bound to an sbp member complementary to an sbp member that comprises a label bound to a nucleotide sequence.

As used herein, a "signal producing system" refers to a system that may have one or more components, at least one component being the label. A signal producing system generates a signal indicative of the presence of a difference between the target polynucleotide sequence and the reference polynucleotide sequence. The signal producing system includes all of the reagents required to produce a measurable signal. When a reporter molecule is not conjugated to a nucleotide sequence, the reporter molecule is normally bound to an sbp member complementary to an sbp member that is bound to or part of a nucleotide sequence. Other components of the signal producing system can include substrates, enhancers, activators, chemiluminescent compounds, cofactors, inhibitors, scavengers, metal ions, specific binding substances required for binding of signal generating substances, coenzymes, substances that react with enzymatic products, enzymes and catalysts, and the like.

As used herein, "ancillary materials" refers to various ancillary materials that frequently may be employed in the methods and assays carried out in accordance with the present invention. For example, buffers will normally be present in the assay medium, as well as stabilizers for the assay medium and the assay components. Frequently, in addition to these additives, proteins may be included, such as albumins, organic solvents such as formamide, quaternary ammonium salts, polycations such as dextran sulfate, surfactants, particularly non-ionic surfactants, binding enhancers, e.g., polyalkylene glycols, or the like.

The phrase "consisting essentially of" when referring to a particular nucleotide or amino acid means a sequence having the properties of a given SEQ ID NO:. For example, when used in reference to an amino acid sequence, the phrase includes the sequence per se and molecular modifications that would not affect the basic and novel characteristics of the sequence.

As used herein, a nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. The term "operably linked" is frequently used to refer to a regulatory sequence capable of mediating the expression of a coding sequence and which is placed in a DNA molecule (e.g., an expression vector) in an appropriate position relative to the coding sequence so as to effect expression of the coding sequence. This same definition is sometimes applied to the arrangement of coding sequences and transcription control elements (e.g. promoters, enhancers, and termination elements) in an expression vector. This definition is also sometimes applied to the arrangement of nucleic acid sequences of a first and a second nucleic acid molecule wherein a hybrid nucleic acid molecule is generated. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. Enhancers do not, however, have to be contiguous to be operably linked. As described herein, PCR amplification may be used to operably link stretches of nucleic acid sequences in a resultant amplicon.

As used herein, the term "oncogene" refers to a genetic sequence whose expression within a cell promotes transformation processes in the cell and/or induces the cell to become a cancerous cell. Similarly, the term "proto-oncogene" is used herein to mean a genetic sequence, residing in the normal genome of a normal, non-cancerous cell, which has the potential, upon additional alterations or in the context of certain cellular milieu, to become an oncogene.

As used herein, a "first portion of a restriction endonuclease site" refers to a stretch of nucleic acid sequences comprising part of a larger stretch of nucleic acid sequences (i.e., restriction endonuclease site) recognized and cleaved by a restriction endonuclease.

As used herein, a "second portion of a restriction endonuclease site" refers to a stretch of nucleic acid sequences comprising part of a larger stretch of nucleic acid sequences (i.e., restriction endonuclease site) recognized and cleaved by a restriction endonuclease.

As used herein, the phrase "a first portion and second portion of a restriction endonuclease site operably linked by a PCR amplification" refers to a generation of a restriction enzyme site comprising a first and second portion, wherein the portions are linked or rendered contiguous by PCR amplification.

As used herein, the term "polymerase chain reaction (PCR) primer pair" refers to a first and a second primer of sufficient complementarily to a template nucleic acid sequence to hybridize to the template nucleic acid sequence at two physically separated sites and on separate strands such that extension from a first primer produces a single stranded nucleic acid that is at least partially complementary to a single stranded nucleic acid extended from a second primer.

As used herein, a "target nucleic acid sequence amplicon" refers to an at least partially double stranded nucleic acid sequence generated by PCR from a target nucleic acid sequence template.

As used herein, a "a pattern of nucleic acid fragments" refers to a plurality of nucleic acid sequences which are produced following, for example, digestion of a larger nucleic acid sequence with a restriction enzyme. Such fragments, therefore, comprise subportions of the larger nucleic acid sequence.

As used herein, "an allele associated with a disorder" refers to an allele comprising a nucleic acid variation or mutation that differs from that of a wild type allele and is associated and/or causatively linked to a disorder.

The basic molecular biology techniques used to practice the methods of the invention are well known in the art, and are described for example in Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York; Ausubel et al., 1988, Current Protocols in Molecular Biology, John Wiley & Sons, New York; and Ausubel et al., 2002, Short Protocols in Molecular Biology, John Wiley & Sons, New York).

Catalogs pertaining to restriction enzymes of utility in the practice of the present invention are known to skilled artisans and a variety of such enzymes are listed in and commercially available through a number of catalogs, including, but not limited to: New England BioLabs 2002-2003 Catalog and Technical Reference, Published by New England Biolabs, 32 Tozer Road, Beverly, MA 01915-9965. Exemplary restriction enzyme sites incorporated into PCR amplicons generated from a template comprising a target nucleic acid sequence comprising a mutation are recognized by Alw26 I, Bcg I, and Sfu I. See Examples I and II.

Protocols pertaining to PCR methodology are provided in a number of laboratory manuals that are known to skilled practitioners and readily available. One such reference useful for the practice of the method of the invention is Goydos and Reintgen. A Molecular Technique Useful in the Detection of Occult Metastases in Patients with Melanoma: RT-PCR Analysis of Sentinel Lymph Nodes and Peripheral Blood. In Methods in Molecular Medicine Series, *Melanoma,* Nickoloff, BJ (ed.) The Humana Press Inc. Totowa, New Jersey 2001.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and described the methods and/or materials in connection with which the publications are cited.

As mentioned above, one aspect of the present invention concerns a method for detecting a difference in the nucleic acid sequence of two related nucleic acid sequences. In the method, the presence of such a difference is detectable by incorporation of a novel restriction enzyme site into PCR amplicons generated by amplification of a sequence comprising a mutation (which may be referred to herein as a target nucleic acid sequence), but not into PCR amplicons generated by amplification of a sequence which does not comprise the mutation (which may be referred to herein as a reference nucleic acid sequence). Amplicons produced following PCR amplification of a plurality of nucleic acid sequence templates comprising target and reference nucleic acid sequences are subsequently digested with an enzyme which recognizes and cleaves the incorporated novel restriction enzyme site of target nucleic acid sequence amplicons. The identification of enzymatic fragments consistent with the presence of target nucleic acid sequence amplicons comprising the novel restriction enzyme site is indicative of the presence of a target nucleic acid sequence in the plurality of nucleic acid sequences. Thus, the method may be employed for detecting the presence of a target nucleic acid sequence in a sample.

Recent evidence suggests that specific defects in components of mitogen-activated protein kinase (MAPK) pathways may be responsible for the pathogenesis of the majority of human melanomas. Davies et al. recently reported that approximately 60% of melanomas have a mutation in *B-RAF* that leads to constitutive activation of MAPK

pathways (Davies et al. Nature 2002; 417(6892):949-54). Mutations in *N-RAS*, an upstream component of this pathway, occur in approximately 25% of melanomas. These *B-RAF* and *N-RAS* mutations appear to be mutually exclusive (Davies et al., *supra*; Ball et al. J Invest Dermatol 1994; 102(3):285-90; Omholt et al. Clin Cancer Res 2002; 8(11):3468-74; Urquhart et al. J Invest Dermatol 2002; 119(3):556-561). Alterations in MAPK signaling may, therefore, play a role in the pathogenesis of the majority of melanomas, implying that the development of drugs that antagonize these changes could lead to improved therapies.

Only a small number of clinical melanoma specimens have been screened for MAPK pathway mutations, however, so the incidence of these mutations in human melanoma is therefore not yet known (Davies et al., *supra*). Accordingly, a clinically useful screening test for the presence of MAPK pathway mutations, which is rapid, sensitive and adaptable to different cell/tissue types that have been maintained and/or preserved by different methods, would be useful for monitoring and stratifying patients undergoing MAPK pathway-targeted therapies.

To address the need for such a clinically useful screening test, the present inventors have developed a sensitive and specific screening assay for the presence of the three most common point mutations observed in molecules involved in MAPK pathways and found to be associated with human melanoma. Exemplary mutations of such MAPK pathway components include, but are not limited to, the V599E mutation of *B-RAF* and the Q61K and Q61R mutations of *N-RAS*. The method of the present invention was used effectively to determine the incidence of these mutations in melanoma specimens. The results presented herein validate the screening method of the invention and document the incidence of *B-RAF* and *N-RAS* mutations in human melanoma.

The method of genetic mutation detection relates detecting mutations in cDNA, reverse transcribed from mRNA extracted from cell lines and snap-frozen tumor tissues. However, in some situations there are not preserved tissue samples to work with and RNA is not be available or has been degraded. Formalin-fixed and paraffin-embedded tissues, for instance, are difficult or often impossible to extract intact RNA from. However, genomic DNA is less likely to be degraded and contains the genetic mutation. The method of the invention works on total genomic DNA extracts from fresh, frozen, or paraffin-embedded tissues.

To demonstrate the generality of the method of the invention, total genomic DNA was extracted from SK-Mel 24, A2058, SK-Mel 2 and MCF-7 cells using a standard

extraction kit (DNA Tissue Kit, Qiagen) and used this genomic DNA in the site-directed mutagenesis detection technique. The presence of the V599E mutation was detected in as few as 10^3 SK-Mel 24 and A2058 cells and obtained no false positive results with genomic DNA from MCF-7 of SK-Mel 2 cells. Next paraffin-embedded melanoma specimens that were obtained from patients who had also donated tumor specimens for flash-freezing and tumor banking were examined. The paraffin-embedded samples were chosen based on the presence or absence of the V599E mutation in the corresponding flash-frozen tumor sample from the same patient. A standard DNA extraction kit was used following the manufacturer's instructions for obtaining genomic DNA from paraffin-embedded samples (DNA Tissue Kit, Qiagen). Positive results were consistently obtained using the site directed mutagenesis detection method in known positive tumor samples and had no false positive results in the tumor samples known not to harbor a V599E *B-RAF* mutation (Figure 6). The forward and reverse Bgl II primers were designed to bind on the same exon so that genomic DNA gave the same size bands as cDNA.

Table 1 provides a list of primer sequences used in the Examples. It is to be understood that the method of the present invention is not limited to the use of the below listed primers.

Table 1

Target	Primer	Sequence (SEQ ID NO:)	Anneal
Beta Actin	Forward	ATGAGATTGGCATGGCTTTA (1)	52° C
	Reverse	GGTGTGCACTTTTATTCAAC (2)	
Tyrosinase	Forward	TTGGCAGATTGTCTGTAGCC (3)	56° C
	Reverse	AGGCATTGTGCATGCTGCTT (4)	
MART-1	Forward	ATGCCAAGAGAAGATGCTCAC (5)	55° C
	Reverse	AGCATGTCTCAGGTGTCTCG (6)	
V599E *B-RAF*	Forward	ATGGATTACTTACACGCCAAGTCAATCATCCACAAAGACC TCA (7)	68° C
	Reverse	CAACTGTTCAAACTGATGGGACCCACTCCATCGAGATGTC (8)	
Q61R *N-RAS*	Forward	ATGACTGAGTACAAACTGGTGGTGGTTGGAGCAGG TGGTGTT (9)	68° C
	Reverse	GCCTGTCCTCATGTATTGCTGTCTCATGGCACTGCACTCTT C (10)	
Q61K	Forward	ATAGATGGTGAAACCTGTTTGTTGGACATACTGGATACAG TTCGA (11)	60° C

Target	Primer	Sequence (SEQ ID NO:)	Anneal
N-RAS	Reverse	CGCTTAATCTGCTCCCTGTAGAGGTTAATATC (12)	
5677insA *BRCA 1*	Forward	GTT GCT ATG GGC CCT TCA CCA ACA TGC CCA CAG ATC AAC T (19)	
	Reverse	CTG CAG TCA GTA GTG GCT GTG GGG GAT CTG GGG TAG GAG G (20)	
185delAG *BRCA 1*	Forward	AAG AAG TAC AAA ATG TCA TTA ATG CTA TGC AGG GAG TCT TAG (21)	
	Reverse	TTT TGC AAA ATT ATA GCT GTT TGC ATA CTC CAA ACC TGT G (22)	
5382insC *BRCA I*	Forward	AAA ACA CCA CAT CAC TTT AAC TAA TCT AAT TAC TGA AGA G (23)	
	Reverse	CCA TAG CAA CAG ATT TCT AGC CCC CTG AAG ATC TTT CCG TCC T (24)	
6174delT *BRCA II*	Forward	CAG TCT CAT CTG CAA ATA CTT GTG GGA TTT TTA CCA CAG C (25)	
	Reverse	ATG GAA ACT TGC TTT CCA CTT GCT GTA CTA AAT CCA GAG A (26)	
5946delCT *BRCA II*	Forward	TGC AAA AAT AAA AAT GCA GCC ATT AAA TTG TCC ATA TCT AAT (27)	
	Reverse	ACC TTA TGT GAA TGC GTG CTA CAT TCA TCA GGA GCT AGA G (28)	
V599E *B-RAF*	Forward	GTTGATTTTGGTCTAGCTACA (29)	

SEQ ID NOs: are indicated by number in parentheses in Table 1. Primers are in 5' to 3' orientation.

EXAMPLE I

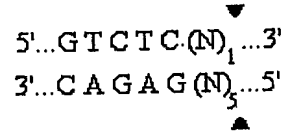
MATERIALS AND METHODS

Detecting Point Mutations in Heterogeneous Tissues: As described herein, a strategy to detect point mutations in heterogenous tissues containing both wild type (WT) and mutant *B-RAF* and *N-RAS* genes was devised. WT mRNA was anticipated to be present in every specimen because clinical melanoma specimens are likely to comprise normal cells (lymphocytes, fibroblasts, etc.), as well as transformed cells, in the tissue samples. Moreover, the melanoma cells are likely to be heterozygous for the *N-RAS* and *B-RAF* alleles. Thus, a method was developed which is capable of detecting a small copy number of mutant *B-RAF* and *N-RAS* alleles in a wild type background.

The method of the invention utilizes reverse transcriptase-polymerase chain reaction (RT-PCR) amplification followed by restriction enzyme digestion to detect point mutations when a mutant allele is present in a sample. As described herein, the methodological strategy involves incorporation of a specific restriction enzyme site into cDNA derived from an allele in which a mutation of interest is present. As a

consequence, the presence of the incorporated restriction enzyme site only in PCR products amplified from mutant allele templates renders such PCR products sensitive to digestion with the restriction enzyme. Accordingly, digestion of the PCR products or amplicons (cDNA) with the restriction enzyme produces a pattern of identifiable fragments of predictable sizes, the appearance of which is indicative of the presence of a mutant allele in a sample. When a sample comprises only a WT allele, the restriction site is not incorporated into the amplified products and the diagnostic pattern of enzymatic fragments is not observed. Of note, restriction sites of the type incorporated in the methods of the invention are not normally present in the Q61R *N-RAS*, Q61K *N-RAS*, or the V599E *B-RAF* mutation sites. As shown herein, however, site-directed mutagenesis using specifically designed PCR primers can be used to introduce changes in sequences located near the point mutations so as to produce diagnostic restriction sites.

Detecting *B-RAF* V599E Mutation: The V599E mutation in *B-RAF* is produced by the substitution of an adenine for a thymine in codon 1796 (A1796T), the incorporation of which results in the substitution of a glutamic acid for a valine at position 599 of the encoded protein. Several restriction sites exist near codon 1796 in *B-RAF*. Alw26 I is a restriction enzyme that cuts the following sequence (arrows indicate the site cut, N = any nucleotide):



The top strand of the Alw26 I site 5'...GTCTC.$(N)_1$...3' is designated herein as SEQ ID NO: 13 and the bottom strand of the Alw26 I site 5'...$(N)_5$GAGAC...3' is designated herein as SEQ ID NO: 14. An Alw26 I restriction site spanning codons 574/575 naturally occurs in *B-RAF*. This restriction site was eliminated by site-directed mutagenesis using a forward primer. See Figure 1A and Table 1. A novel, diagnostic Alw26 I restriction was introduced into the V599E mutant *B-RAF* sequence by site directed mutagenesis using a reverse primer. See Figure 1A and Table 1. These primers produced a 160 bp product that was cut by Alw26 I into 123 bp and 37 bp fragments if the V599E mutation was present, but remained a 150 bp product if only the WT sequence was present. See Figure 1B. The digested PCR product was run on a 15% polyacrylamide gel and visualized using ethidium bromide staining. See Figure 1C.

In order to increase the length of the fragments for Southern Blotting detection, the primer design was changed to produce a 310bp amplicon that contains a Bgl II restriction site when the V599E mutation is present. Bgl II cleaves the following sequence:

5′ A^G A T C T 3′
3′ T C T A G^A 5′

The top and bottom strand of the Bgl II site area identical and are designated herein as SEQ ID NO : 18. The arrows indicate the location of cleavage that leaves a 4 base overhang. The reverse primer introduced changes in the amplicon at the 3rd and 4th bases from the 3' end; two adenines were replaced with a thymine and a cytosine to produce a Bgl II site in the V599E mutant sequence (Figure 9A). These primers produced a 310bp amplicon that was digested into 270bp and 40bp fragments in the presence of Bgl II. Fragments of this size have been easier to detect on Southern Blotting (Figure 9D).

Detecting *N-RAS* Q61R and Q61K Mutations: In that the two most common point mutations that result in constitutive activation of *N-RAS* in melanoma (Q61R and Q61K) do not form unique restriction sites, site-directed mutagenesis was used to produce restriction sites that could be used to advantage to identify these *N-RAS* mutations when present in a sample. Using site-directed mutagenesis of codon 64 of *N-RAS*, a Bcg I restriction site was incorporated specifically into PCR products amplified from mutant alleles comprising the Q61R mutation. The Bcg I site was not, however, incorporated into PCR products amplified from either WT alleles or alleles comprising the Q61K mutation. See Figure 2A and Table 1. As shown below, Bcg I cuts the following sequence (arrows indicate the areas cut, N=any nucleotide):

5'... ▼NNN NNN NNN NNN CGA NNN NNN TGC NNN NNN NNN N▲ ...3'

The Bcg I site 5'...NNN NNN NNN NNN CGA NNN NNN TGC NNN NNN NNN N...3' is designated herein as SEQ ID NO: 15. Digestion of PCR products amplified from alleles which do not comprise the Q61R mutation with Bcg I produced a 34bp fragment, and cut at the 5' and 3' ends of the cDNA molecule. In contrast, digestion of PCR products amplified from Q61R alleles into which specific primers had been used

to insert a nucleotide change, resulted in the generation of 168bp, 34bp, and 22bp Bcg I cleaved fragments of the 224bp PCR product. See Figure 2B and 2C.

To detect the Q61K mutation, an Sfu I restriction site was incorporated specifically into cDNA sequences comprising the Q61K mutation by using primers that change nucleotides in codons 59 and 60 of *N-RAS*. See Figure 3A. As shown below, Sfu I cuts the following sequence (arrows indicating the area cut):

5'...T T C G A A...3'

The top strand of the Sfu I site 5'...TTCGAA...3' is designated herein as SEQ ID NO: 16. Primers that insert the two nucleotide changes shown in Figure 3A produce a 250bp PCR product that is cut by Sfu I into 210bp and 40bp fragments if the Q61K mutation is present in *N-RAS*. See Figure 3B and 3C.

Sensitivity and Specificity: To test the sensitivity and specificity of this technique for identifying the V599E mutation, plasmids were constructed that contained either the WT *B-RAF* sequence or the V599E mutant sequence. Primers were designed that amplified a 300 bp segment of *B-RAF* cDNA centered around codon 1796. MCF-7 cells (known to be *B-RAF* WT) (Davies et al. *supra*) were used to produce the cDNA for the WT plasmid and HT-144 melanoma cells (known *B-RAF* V599E mutant) (Davies et al. *supra*) were used to produce the cDNA for the mutant plasmid. These cDNAs were inserted into plasmids according to the manufacturer's protocol (Invitrogen). Plasmids were cloned and amplified in competent *E. coli*, and were sequenced using the sequencing primers supplied with the plasmid to verify the sequence. To determine the sensitivity and specificity of the V599E detection protocol, B-RAF sequence containing plasmids were serially diluted from 10^9 to 10 copies/reaction and these diluted solutions were used in the V599E detection protocol.

The sensitivity and specificity of the system for detecting *N-RAS* mutations was corroborated using essentially the same method as described above for detecting the *B-RAF* mutation. Briefly, plasmids were constructed that comprised either a WT *N-RAS* cDNA or one of the Q61 mutations. These N-RAS-containing plasmids were serially diluted from a concentration of 10^9 down to 10 copies/reaction. Plasmids comprising either WT or mutant sequences were also used in all experiments as positive and negative controls.

Needle aspiration: Fine needle aspirations (22 gauge needle) of known V599E mutant tumor specimens were obtained and the tumor cells extracted by this aspiration were used in the site-directed mutagenesis reaction. Positive results from these fine-needle aspirants were obtained consistently (Figure 10), demonstrating the utility of this method in the clinical setting when a fine-needle or bone marrow aspirant may be the only tumor sample available.

Western blotting: Cultured cells or homogenized frozen tissue specimens were lysed on ice for 45 minutes with RIPA buffer (10 mM sodium phosphate, pH 7.2, 1% nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA) supplemented with fresh 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 50 μg/ml leupeptin, and centrifuged at 14,000 x g at 4°C for 10 minutes. Proteins were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. The blots were incubated in blocking solution consisting of 5% milk in TBS-T (0.1% Tween-20) for 1 hour at 25°C, then immunoblotted with polyclonal anti-ERK antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-phospho-MEK antibody (Sigma, St Louis, MO). Detection by enzyme-linked chemiluminescence was performed according to the manufacturer's protocol (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Experiments were done under conditions of linearity with respect to protein concentration.

Clinical Melanoma Specimens: 115 melanoma samples were analyzed herein, which included primary tumors (36), regional dermal metastases (27), nodal metastases (40), and distant metastases (12). The Tissue Retrieval Service of the Cancer Institute of New Jersey banked these specimens, under a blanket IRB approved protocol, in a fashion that preserves patient confidentiality and conforms to HIPAA regulations. Each specimen was obtained in the operating room and snap frozen in liquid nitrogen within 5 minutes of resection. The specimens were stored at –80° C until processed. Total RNA was extracted from the specimens using a standard spin-column based system (RNAeasy, Qiagen) and first-strand synthesis was performed using a standard kit (Superscript First Strand Synthesis Kit, Invitrogen). All melanoma specimens used in this study were positive by PCR for beta actin (Ponte et al. Nucleic Acids Res 1984; 12(3):1687-96), tyrosinase (Brossart et al. J. Invest. Dermatol. 1993; 101(6):887-889; Goydos et al. J Am Coll Surg 1998; 187(2):182-190) and MART-1 (Goydos et al. *supra*; Reed and Albino. Clin Lab Med 2000; 20(4):817-38), the presence of which, respectively, confirmed RNA integrity and the presence in the specimen of melanoma cells.

Primers and PCR Conditions: The primers and PCR conditions used for the detection of beta-actin, tyrosinase, and MART-1, as well as the primers used in the site-directed mutagenesis detection protocol for the V599E *B-RAF* and the Q61 *N-RAS* mutations are shown in Table 1. PCR reactions (25µl reactions) were run using a standard *Taq* polymerase (MasterTaq, Eppendorf), with an initial denaturing step at 94°C for 3 minutes followed by 40 cycles consisting of denaturing at 94°C for 30 seconds, annealing at the specific temperature for each set of primers for 30 seconds, and extension at 72°C for 1 minute. A final extension at 72°C for 8 minutes was followed by storage at 4°C. Restriction enzyme digestions were performed using 10µl of the resultant PCR product at 36°C for two hours with the appropriate enzymes and buffers as per manufacturer's instructions. Digests were run on 15% polyacrylamide gels and visualized by ethidium bromide staining and UV transillumination. Controls included the use of known mutant and wild-type specimens in each run. BLAST searches confirmed that the primer sequences used in these reactions did not anneal to other known genes.

Statistical Analysis: The statistical analysis was performed at the Division of Biometrics at The Cancer Institute of New Jersey. Comparison of the incidence of the V599E point mutation in melanoma samples from different stages of progression was performed using contingency tables and the chi squared test, with P values less than 0.05 considered significant.

Specific Methodological Details Pertaining to the Drawings

In Figure 1A, an arrow indicates the thymine to adenine V599E point mutation. Changes introduced by the primers are shown in boxes. An adenine is substituted for guanine near the 3' end of the forward primer to eliminate an Alw26 I site that spans exons 574 and 575 in both the mutant and WT sequences. A substitution of a cytosine for an adenine in the reverse primer produces a new Alw26 I restriction site in the mutant sequence but not in the WT sequence. Figure 1B shows the resultant digestion products. Figure 1C shows a representative agarose gel electrophoresis result. L=ladder, Pos = positive control (HT-144 cell line), Neg = Negative control (MCF-7 cell line), lanes 1 - 5 are tumor samples. Tumor sample 3 shows cutting with Alw26 I demonstrating a V599E mutation of *B-RAF* in this tumor sample. A 160bp band is still seen after digestion of the PCR product from tumor sample 3 and most likely represents WT *B-RAF* sequence from contaminating normal cells in that sample.

In Figure 2A, the 3' end of the reverse primer introduced a change from a guanine to an adenine (box) in codon 64 to produce a Bcg I restriction site in the Q61R mutant cDNA that is not present in the WT or Q61K mutant cDNA. The forward primer binds to the first 40 nucleotides of the cDNA to produce a 224 bp PCR product that is cut by Bcg I into a 168 bp product, a 34 bp product, and a 22 bp product if the sequence contains the Q61R mutation, but remains as a 224 bp product if the sequence contains the Q61K mutation (see Figure 4) or is WT. Figure 2A shows the resultant digestion products. Figure 2C shows a representative agarose gel electrophoresis result. L=ladder, lane 1 = positive control (SK-Mel 2 cell line), lane 2 = negative control (MCF-7 cell line), lanes 3 – 7 are tumor samples. Tumor samples 5 and 6 show cutting with Bcg I demonstrating a Q61R mutation of *N-RAS* in these two tumor samples (34 bp band is very faint at bottom of gel and 22 bp band is not shown). A 224bp band was still seen after digestion of the PCR product from tumor samples 5 and 6 that most likely represented WT *N-RAS* sequence from normal cells present in the sample.

In Figure 3A, the 5' end of the forward primer introduces a change of a thymine to a cytosine and a cytosine for a guanine (boxes) in codons 59 and 60 to produce an Sfu I restriction site in the Q61K mutant that was not present in the WT or Q61R mutant. The reverse primer binds downstream to produce a 250 bp PCR product that is cut by Sfu I into a 210 bp product and a 40 bp product if the sequence contains the Q61K mutation but remains as a 250 bp product if the sequence comprises the Q61R mutation or is WT. Figure 3B shows the resultant digestion products. Figure 3C shows a representative agarose gel electrophoresis result. L = ladder, Neg = negative control (MCF-7 cell line), Pos = positive control, lanes 1 - 9 are tumor samples. Tumor sample 9 shows cutting with Sfu I demonstrating a Q61K mutation of *N-RAS* in this tumor sample (40 bp band is not shown). A 250bp band is still seen after digestion of the PCR product from tumor sample 9 that most likely represents WT *N-RAS* sequence from contaminating normal cells in that sample.

In Figure 4, serial dilutions of plasmids range from 10^6 to 10 copies per reaction. L = Ladder, 0 = no plasmid in the reaction. A 123 bp band is visible at a concentration as low as 10^2 plasmids per reaction.

Figure 5A shows a Western blot of protein samples extracted from cell lines with either WT *N-RAS* and *B-RAF* or a mutation in one or the other of these genes. Antibodies against ERK 1/2 (upper panel) or MEK 1/2 (lower panel) were used. SK-Mel 31 melanoma cells (lane 2) and HUVEC cells (lane 6) are known to be WT for both *N-RAS*

and *B-RAF* and are used as the baseline level of phosphorylated MEK and ERK. A2058 melanoma cells (lane 3), HT-144 melanoma cells (lane 4), and MEL 501 melanoma cells (lane 5) have known V599E *B-RAF* mutations and showed varying degrees of increased levels of phosphorylated MEK and ERK over baseline. SK-Mel 2 cells (lane 1) have a known *N-RAS* mutation (Q61R) and showed a similar increase in the phosphorylated forms of MEK and ERK as the cell lines with V599E *B-RAF* mutations. Arrows indicate the two bands representing ERK1 and 2 (upper panel) and the two bands that represent MEK1 and 2 (lower panel). Loading controls were run demonstrating equal protein loading in each lane (not shown).

Figure 5 B shows a Western blot using protein extracted from 7 human melanoma specimens. Tumors 1-3 were found to be WT in both B-Raf and N-Ras and show little increase in the phosphorylated forms of ERK 1 and 2. Tumors 4 and 5 were found to harbor the V599E B-Raf mutation and each showed increased phosphorylation of ERK 1 and 2. Tumor 6 harbors a Q61K N-Ras mutation and Tumor 7 harbors a Q61R N-Ras mutation and both demonstrated an increase in the phosphorylated forms of ERK 1 and 2. Lane 8 is a negative control (MCF-7 cell line) and lane 9 a positive control (HT-144 cell line, known V599E mutant). Total ERK 1 and 2 staining demonstrates that increases in total ERK 1 and 2 did not account for the increase observed in the phosphorylated forms.

RESULTS

Determining the Sensitivity and Specificity of Site-directed Mutagenesis: To determine sensitivity and specificity of the site-directed mutagenesis procedure on plasmids containing either mutant or WT *B-RAF* cDNA, solutions comprising these cDNAs were serially diluted from 10^9 to 10 copies/reaction and tested as described herein. Figure 4 demonstrates that as few as 100 copies of mutant *B-RAF* sequence were detectable using the method of the invention. Moreover, up to 10^{11} copies per reaction of WT sequence produced no detectable mutant-specific bands when digested with Alw26 I (data not shown). Using the same method, plasmids that contained either a WT *N-RAS* cDNA or one of the Q61 mutations were tested, and as few as 100 copies of either the Q61R or the Q61K mutation were detectable. Furthermore, no detectable mutant-specific band was produced when amplification products generated from as many as 10^{11} copies of WT sequence PCR template per reaction were digested with Sfu I or Bcg I (data not shown).

Incidence of *B-RAF* and *N-RAS* Mutations in Melanoma: The site-directed mutagenesis technique of the present invention was used to detect V599E *B-RAF* mutations and the Q61K and Q61R *N-RAS* mutations in 115 clinical melanoma samples. As shown herein, 89 of the 1185 samples (25 of 36 primary tumors, 18 of 27 regional dermal metastases, 16 of 40 nodal metastases, and 9 of 12 distant metastases) had the V599E mutation (60 %). None of the samples positive for a V599E mutation comprised either a Q61K or Q61R *N-RAS* mutation. Of the 115 samples tested, 21 (18%) contained an *N-RAS* mutation (4 primary tumors, 5 regional dermal metastases, 10 nodal metastases, and 2 distant metastasis), 17 contained a Q61R mutation and 4 contained a Q61K mutation. Thus, either an *N-RAS* or *B-RAF* mutation was detected in 89 of 115 samples tested (77%). Twenty-six of the 115 melanoma samples analyzed herein contained neither the Q61 *N-RAS* mutation nor the V599E *B-RAF* mutation (23 %). There was a significant difference in the incidence of the V599E B-RAF mutation in melanoma samples representing different stages of disease progression. Indeed, regional dermal metastatic lesion and distant metastases had a significantly higher incidence of the V599E mutation than that observed for primary tumors or nodal metastatic lesions ($p<0.01$).

Downstream Effects of Activating MAPK Mutations in Cell Lines: Anti-phospho - MEK and ERK antibodies (Kolch. Biochem J 2000; 351 Pt 2:289-305; Lee and McCubrey. Leukemia 2002; 16(4):486-507; Marais and Marshall. Cancer Surv 1996; 27:101-25; Peyssonnaux and Eychene. Biol Cell 2001; 93(1-2):53-62) were used to detect MAPK activation in cell lines known to be WT for *N-RAS* and *B-RAF* (HUVEC cells, and SK-Mel 31 melanoma cells; Davies et al. *supra*), mutant in *N-RAS* (SK-MEL 2 melanoma cell line; Davies et al. *supra*), or mutant for the V599E *B-RAF* mutation (HT-144, Mel 501, and A2058 melanoma cell lines; Davies et al. *supra*). The level of phosphorylation of MEK and ERK in WT cell lines examined was considered the baseline level. As shown in Figure 5A, mutant cell lines had different elevated expression levels of phosphorylated MEK and ERK as compared to those of WT cell lines.

Downstream Effects of Activating MAPK Mutations in Clinical Specimens: The above-mentioned anti-phospho-ERK antibodies were also used to detect MAPK activation in clinical melanoma specimens. See Figure 5B. As shown herein, relative increases in phosphorylated forms of ERK in melanoma samples comprising V599E *B-RAF* or one of the two Q61 *N-RAS* mutations were observed as compared to those of specimens comprising only WT alleles.

DISCUSSION

As described herein, 39% of the clinical melanoma specimens tested harbored a V599E *B-RAF* mutation and 20% harbored one of the two known Q61 *N-RAS* mutations, thus demonstrating that approximately 60% of melanomas comprise one of these mutations. The incidence of *N-RAS* mutations in this study corroborated results described in previous reports (Ball et al. J Invest Dermatol 1994; 102(3):285-90). The incidence of the V599E *B-RAF* mutation presented herein was lower than reported by Davies et al who found that 5 of 9 melanoma specimens and 11 of 15 short-term melanoma cultures contained the V599E mutation (Davies et al. *supra*). The present analysis, however, involves a significantly larger number of melanoma specimens, and includes specimens from different states of disease progression. Thus, the present study presents a more comprehensive and, therefore, predictive assessment of the association/correlation of the presence of such mutant alleles with melanoma and disease progression.

As described herein, primary tumors (7 of 25) and nodal metastasis (5 of 25) had a lower incidence of the V599E mutation than did regional dermal metastases (15 of 25) or distant metastases (6 of 10) ($p<0.01$). These data suggest that MAPK pathway mutations may be found at different rates in tumors representing different stages of progression and indicate that the different mutations impart different characteristics to a melanoma cell. Moreover, sixty percent of distant metastatic lesions in the present study contained a V599E mutation and 20% contained an N–RAS mutation, thereby suggesting that distant metastases have the highest incidence of mutations in MAPK pathway components.

These data were corroborated by the observation of an increase in the phosphorylation levels of downstream components/molecules of the MAPK pathway in transformed cells. As shown herein, a significant increase in phosphorylation levels of MEK and ERK was observed in cell lines and melanoma specimens that comprised one of the MAPK mutations (see Figure 5), suggesting that functional up-regulation of this pathway (Kolch. Biochem J 2000; 351 Pt 2:289-305; Lee and McCubrey. Leukemia 2002; 16(4):486-507; Marais and Marshall. Cancer Surv 1996; 27:101-25; Peyssonnaux and Eychene. Biol Cell 2001; 93(1-2):53-62) occurs in the MAPK mutated specimens.

The results presented herein suggest that activating mutations in the MAPK pathway play a prominent role in the development of melanoma. Therefore, targeting this pathway with specific therapeutic regimens is an important clinical goal. Clinical trials will require a specific and sensitive method to detect these mutations. Sequencing to

detect such mutations, however, is expensive, time consuming, and requires sophisticated equipment and trained staff and personnel. Single-strand conformation polymorphism (SSCP) and denaturing gradient gel electrophoresis (DGGE) are two other methods used to detect gene mutations in tumor specimens. The sensitivity and specificity of SSCP and DGGE, however, are questionable and the necessity of confirming the presence of the mutation by sequencing limits the usefulness of these methods (Campos et al. Clin Chem Lab Med 2001; 39:401-404; Nedergaard et al. Int J Cancer 1997; 71:364-369; Pogue et al. Genomics 1998; 54:1-4). Detecting the mutant protein with specific antibodies using either immunohistochemical or Western blotting techniques is also useful. Immunohistochemistry is particularly useful for the simplified screening of archival, paraffin-embedded specimens. Immunohistochemical and immunoblotting approaches are limited, however, by the availability of diagnostic antibodies and technical expertise required to perform the assays accurately.

Herein is described a new method for screening to detect MAPK mutations which is based on the creation of a new diagnostic restriction site in amplicons generated by PCR amplification of either mutant B-RAF and N-RAS alleles. The method of the present invention is novel, sensitive, specific, simple, and inexpensive. Moreover, multiple samples can be screened readily, thus lending the methodology to high throughput screening approaches. Such qualities render this method suitable for use in analysis of clinical specimens, in which tissue is limited and comprises a mixture of tumor and normal cells.

Treatment protocols that target MAPK pathway components have been examined in many different tumor types (Lee and McCubrey. Leukemia 2002; 16(4):486-507; Lyons et al. Endocr Relat Cancer 2001; 8(3):219-25; Midgley and Kerr. Crit Rev Oncol Hematol 2002; 44(2):109-20; Nottage and Siu. Curr Pharm Des 2002; 8(25):2231-42; Sebolt-Leopold. Oncogene 2000; 19(56):6594-9). Many of the methods that have been used to target this pathway in other cancer types may soon be tested in patients with melanoma. It will thus be important to be able to stratify patients by MAPK component mutation status in these protocols. The site-directed mutagenesis method of the present invention addresses this need.

EXAMPLE II

The method of the present invention, which utilizes a modified site-directed mutagenesis approach to detect mutations in heterogeneous tissues, may also be used to

advantage to detect mutations in archival tissues. For such applications, genomic DNA from the sample is utilized as template for PCR amplification. The ability to use the method of the present invention in the context of archival tissue (e.g., paraffin-embedded tissue) greatly expands the utility of the method because large banks of such material are available for analysis. Moreover, DNA is much more stable than RNA and can survive intact in paraffin-embedded specimens.

A: The method of the present invention was used to analyze nucleic acid sequences isolated from paraffin-embedded melanoma specimens. The paraffin-embedded melanoma tumor samples analyzed were prepared in parallel from the same tissue samples that were flash-frozen and screened for MAPK pathway point mutations in Example I. Thus, the results produced using archival tissue as starting material are corroborated by and are corroborative of those results generated using flash frozen tissue.

Briefly, paraffin-embedded tumor samples were dewaxed using a standard method involving elevated temperature that is well known to those of skill in the art. Dewaxed tissue was placed in an Eppendorf tube and digested with proteinase K at 56°C overnight. Genomic DNA was extracted from the digested tissue with phenol and precipitated with ethanol in the presence of glycogen. PCR was performed using total genomic DNA and the Alw26 I site-directed mutagenesis primers described hereinabove. The resultant PCR products (i.e, amplicon) were cut with Alw26 I at 37°C for two hours and analyzed on a 15% polyacrylamide gel, which was stained with ethidium bromide to visualize the nucleic acid sequence fragments present in the PCR mix. For this analysis, melanoma samples known to be wild type for B-Raf (comprising only reference nucleic acid sequence) and samples known to comprise the V599E mutation (target nucleic acid sequence) were tested. In brief, the method of the invention was used successfully to detect the V599E mutation in all of the positive samples and did not register any false positive results for the samples known to be wild type at the codon encoding amino acid residue 599. Thus, the method of the present invention may be used to advantage for detecting mutations in nucleic acid sequences derived from archival tissue.

B: PCR was carried out on genomic DNA extracted from four different paraffin-embedded tumor samples using site directed mutagenesis primers that produce a Bgl II restriction site in sequences containing the V599E mutation. 10µl of PCR product was digested with Bgl II at 37°C for 2 hours and 12 µl of this product was loaded in each lane. Figure 6 shows a picture of an agarose gel of the digested PCR products following

electrophoresis. Lanes 1 and 3 are known V599E mutant tumors and Lanes 2 and 4 are known *B-RAF* WT tumors. The 40 bp band is not shown.

EXAMPLE III

The method of the present invention has been modified to address applications in which an overwhelming excess of wild type nucleic acid sequences in a sample renders detecting a small number of nucleic acid sequences comprising a mutation difficult. The excess of wild type nucleic acid sequences in the sample appears to decrease the level of amplification of the minority mutated nucleic acid sequences. To increase the sensitivity of the method of the invention under such conditions, a two-step method was developed.

In the first step, the amount of wild type amplicon/cDNA produced by PCR (i.e., cDNA generated by amplification from a wild type template) was reduced by selective digestion using a second restriction enzyme. To achieve this end for samples wherein detection of the V599E *B-RAF* mutation was the objective, a reverse primer was designed such that its incorporation not only produced a new Alw26 I restriction site in mutant amplicons, but also a new Tsp45 I site in wild type amplicons. Tsp45 I cuts the following sequence (arrows indicate area cut, S = C or G):

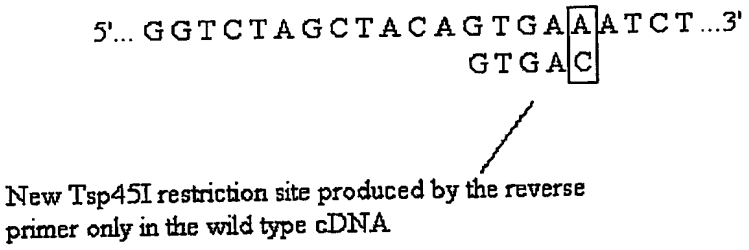
5'... G T S A C ...3
3'... C A S T G ...5'

The top and bottom strand of the Tsp45 I site are identical 5'...GTSAC...3' and are designated herein as SEQ ID NO: 17. As illustrated above, the original reverse primer used to produce the Alw26 I site in amplicons generated from the V599E mutant *B-RAF* produced a Tsp45 I restriction site in wild type *B-RAF* amplicons. See diagram hereinbelow. To capitalize on this feature, a forward primer that binds upstream relative to that of the binding site for the forward primer of the *B-RAF* sequence described in Example I was designed. When used with the original Alw26 I reverse primer, this forward primer produces a 407 bp cDNA/amplicon. Amplicons generated from wild type nucleic acid sequence templates are digested by Tsp45 I to generate fragments of 365 bp and 42 bp, whereas amplicons generated from mutant nucleic acid sequence templates were not digested by Tsp45 I and remained intact (407 bp). See diagram hereinbelow.

In brief, PCR amplifications were performed using the Tsp45 I forward and Alw26 I reverse primers to produce amplicons that were subsequently digested with

Tsp45 I at 65°C for 2 hours. This initial digestion cuts the wild type amplicons/cDNA into smaller segments, and yet preserves V599E mutant amplicons, thus providing a means to select for mutant amplicons as intact and functional templates for a second PCR amplification. A second PCR was subsequently performed using the Tsp45 I digested PCR products as templates for extension/amplification and the Alw26 I forward and reverse primers. The fragmented wild type amplicons were not amplified by this second PCR reaction, allowing for selective amplification of the intact V599E mutant amplicons. Using this two-step technique, as few as 500 cells comprising V599E mutant encoding nucleic acid sequences were detectable, even when admixed with 10^6 wild type cells. Similar two-step methods have been developed to detect mutant Q61R and Q61K *N-RAS* mutant cells in the face of overwhelming excess of wild type cells.

Diagrammatic representation of this exemplification of the invention:



EXAMPLE IV

Detecting BRCA Mutations: The mutations were detected using the method described in Example I. Using the site-directed mutagenesis method, five mutations were chosen at random and included the 185delAG, 5677insA and 5381insC *BRCA* 1 mutations and the 6174delT and 5946delC *BRCA* 2 mutations. Primers sets were designed to insert new restriction sites in the mutant amplicons produced during PCR amplification of the cDNA from cell lines containing these mutations. The 5 *BRCA* were routinely detected using the site-directed mutagenesis method with only two enzymes (Bsl I and BsaXI) and a single universal buffer (see Figures 7A, 7B and 8). In particular each mutation was amplified using specific site directed mutagenesis primers (Table 1) and standard PCR in 25µl reactions. 5 µl of the amplicon was digested with a specific restriction enzyme for two hours and then sized fractionated on a 2% agarose gel. The bands were visualized using ethidium bromide staining and UV transillumination.

Restriction enzyme digestion was carried out in a universal buffer (New England Biolabs buffer number 4). The restriction enzyme BsaXI (New England Biolabs) was used to digest the 185delAG, 5946delCT, and 5677insA amplicons while Bsl I (New England Biolabs) was used to digest 6174delT and 5382insC amplicons. The digestion results are shown in Figures 7A and 7B.

Figure 7A shows a Bsl I restriction site near codon 1982 of the *BRCA* 2 gene containing a 6174delT mutation. The arrow indicates the location of the deleted thymine that results in the mutation. The forward primer introduces a change of a cytosine for a guanine in codon 1979 that results in a Bsl-I restriction site in the mutant sequence absent in the wild-type sequence.

Figure 7B: shows the resultant digestion products.

The Bsl I enzyme and a different set of primers were used to detect the 5382insC *BRCA1* mutation (Figure 8). Sspecific primer sets and the enzyme BsaXI were used to detect the 185delAG and 5677insA *BRCA1* mutations and the 5946delCT *BRCA2* mutation. BsaX1 cuts the 300bp amplicons into 245bp, 30bp, and 25bp fragments (schemas not shown)

Figure 8 shows five different *BRCA* 1 and 2 mutations from cell lines. Each mutation was amplified using specific site-directed mutagenesis primers and standard PCR in 25µl reactions. 5µl of the amplicon was digested with a specific restriction enzyme for two hours and then size fractionated on a 2% agarose gel. The bands were visualized using ethidium bromide staining and UV transillumination. Restriction enzyme digestion was carried out in a universal buffer. The restriction enzyme BsaX I (New England Biolabs) was used to digest the 185delAG, 5946delCT, and 5677insA amplicons while Bsl I (New England Biolabs) was used to digest 6174delT and 5382insC amplicons. 30bp, 25bp, and 40bp bands are not shown. A 300bp band is still present after enzymatic digestion because the cell lines (supplied by Corriell Institute for Medical Research) contain heterozygous *BRCA* alleles. MCF 7 breast cancer cells were used as a control and amplification and enzymatic digestion of the *BRCA* genes using cDNA from this *BRCA* wild type cell line resulted in no cutting of the amplicon using any combination of primers and restriction enzymes (data not shown).

EXAMPLE V

Breast Cancer—Detecting mutations in individual tissues: Each tissue specimen is flash-frozen in liquid nitrogen and stored in liquid nitrogen until processed. 300mg of

tissue is minced with a scalpel and mixed with 20µl of proteinase K and 200 µl of buffer, and vortexed to mix. The mixture is placed at 70°C for 30 minutes. 200 µl of 100% ethanol is added and the mixture is vortexed to mix. The mixture is pipetted onto a spin column and centrifuged at 6000 x g for one minute. The column is washed and the total genomic DNA eluted in a stabilizing buffer by centrifugation. PCR is performed using the genomic DNA, the appropriate site-directed mutagenesis primers, Taq polymerase, buffer, and dNTPs in a standard thermocycler. Five microliters of the resultant PCR product is digested with the appropriate restriction enzyme for 2 hours at 37°C and the digestion fragments run on ethidium bromide stained agarose gels and visualized by UV transillumination.

Assay Development: 45 different primer pairs relating to 45 known mutations are prepositioned in separate wells of 96 well PCR plates, the plates are sealed, and stored at –20°C until needed. To screen a sample a PCR master mix is produced consisting of Taq polymerase, buffer, dNTPs, and DNA from the tissue sample. This master mix is aliquoted into the primer containing wells of the 96 well plate to produce 25µl reaction mixes in each well and the PCR reaction is run using the universal annealing temperature. Using a multichannel pipetter 5µl of the PCR reaction is transferred from each well to the wells of a second 96 well plate that contains the restriction enzymes and universal buffer mix. This transfer will produces a 20 µl restriction digestion reaction. This second plate is incubated for two hours at 37°C. Gel loading buffer is added to each well and the multichannel pipetter is used to transfer 15µl of the digestion reaction plus loading buffer mix to the wells of a 2% ethidium bromide containing agarose gel for size fractionation. With 45 primer and restriction enzyme combinations each reaction is run in duplicate on a single 96 well plate. A positive result is defined as digestion of the PCR amplicon that results in the proper sized band visualized on the agarose gel by UV transillumination [23] in both of the duplicate reactions. If only one reaction of the duplicates results in a positive result the reaction is repeated using that specific primer and restriction enzyme combination to confirm the positive result. Controls are run with each assay consisting of cDNA from known *BRCA* mutant cells to confirm PCR amplification and that each enzyme is functioning properly. Using a 96 well plate 45 samples are run in duplicate and 6 control wells.

EXAMPLE VI

The presence of mutations in the EGFR in non-small cell lung cancer is determined using the process set forth in Example 1 wherein the primers are designed to produce new restriction sites in PCR amplicons containing the specific mutation but not in wild type amplicons. Detection of these mutations is performed by specific restriction enzyme digestion of the amplicons and visualization of the digestion fragments on ethidium bromide-stained agarose gel electrophoresis and UV transillumination. Genomic DNA is used for the PCR template or mRNA is reverse transcribed to cDNA and this cDNA is used as the template for these experiments. One example of such a reaction would be the extraction of genomic DNA from paraffin-embedded non-small cell lung cancer specimens. PCR is performed using this DNA template, site-directed mutagenesis primers that insert a new restriction site in PCR amplicons containing the mutation we are searching for, Taq DNA polymerase, buffer, and dNTPs in a 25μl PCR reaction. The resultant PCR amplicon is digested with the appropriate restriction endonuclease and digestion fragments visualized by ethidium bromide-stained agarose gel electrophoresis and UV transillumination. Detecting the correct digestion fragments confirms the presence of the mutation in the tissue. Detecting no digestion of the PCR amplicon confirms that the specific mutation being sought is not present in the tissue specimen.

EXAMPLE VII

The presence of mutations in the *K-RAS* gene in pancreatic cancer is determined using the process set forth in Example 1 wherein the primers are designed to produce new restriction sites in PCR amplicons containing the specific mutation but not in wild type amplicons. Detection of these mutations is performed by specific restriction enzyme digestion of the amplicons and visualization of the digestion fragments on ethidium bromide-stained agarose gel electrophoresis and UV transillumination. Genomic DNA is used for the PCR template or mRNA is reverse transcribed to cDNA and this cDNA is used as the template for these experiments. One example of such a reaction is the extraction of tumor-cell containing fluid from the duodenum of patients with pancreatic cancer. Genomic DNA is extracted from these cells and PCR performed as described above. The resultant PCR amplicon is digested with the appropriate restriction endonuclease and digestion fragments visualized by ethidium bromide-stained agarose

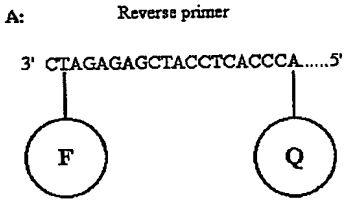
gel electrophoresis and UV transillumination. Detecting the correct digestion fragments confirms the presence of the mutation in the tissue. Detecting no digestion of the PCR amplicon confirms that the specific mutation being sought is not present in the tissue specimen. Detecting *K-RAS* mutations in cells found in the duodenum of patients is a way to diagnose pancreatic cancer in these patients.

EXAMPLE VIII

Fluorescence based detection:

Reducing background fluorescence: An oligonucleotide having the sequence of SEQ ID NO: 29 was bound to the fluorophore Fluorescein dT and a biotin-labeled oligonucleotide complementary to it was prepared by means known in the art. 10 picomoles of the fluorophore-bound oligonucleotide were placed in 25µof water and the fluorescence was measured in a standard bench top fluorometer. Then 30 picomoles of the biotin-labeled complementary oligonucleotide was added and the mixture was incubated at 52° for ten minutes then cooled to room temperature. Strepavidin coated metallic beads (MagneSphere, Promega, Madison, WI) were added to the mixture and the mixture was incubated at 25° for 10 minutes. The metallic beads were removed from the solution using a magnetic stand and the supernatant was transferred to a clean tube. The fluorescence of the supernatant was measured and a 95% decrease in fluorescence in the mixture was found; the initial reading was 1510 RFU (relative fluorescent units) that dropped to 95 RFU after extraction.

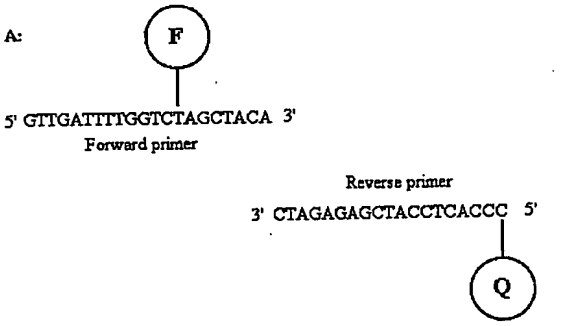
Dual-labeled reverse primer method: A primer such as



is designed placing the quencher on the 5' end of the oligo and the fluorophore near the 3'end. In the Bgl II reverse primer this will place the fluorophore at the thymine one base back from the 3' end. In the Bcg-1 reverse primer the fluorophore is placed at the thymine

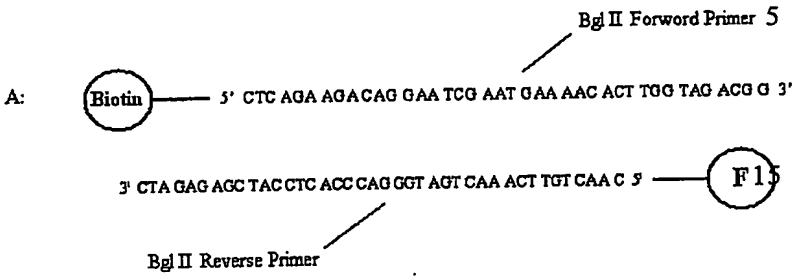
6 bases back from the 3' end, in the 34bp fragment. The reverse primers for both the Bgl II and Bcg-1 reactions contain Iowa Black version 1.0 as the quencher at the 5' end and Fluorescein dT as the fluorophore (IDT Technologies). The Bgl II forward primer binds more proximally along the *B-RAF* cDNA to produce a 300bp amplicon cut by Bgl II into 270bp and 30bp fragments if the V599E mutation is present but remains a 200bp oligonucleotide if the gene is wildtype. The Bcg-1 forward primer binds more proximally along the *N-RAS* cDNA to produce a 224bp amplicon cut by Bcg-1 into 164bp, 34bp, and 22bp fragments if the Q61R mutation is present but remain a 224bp oligonucleotide if the gene is wildtype.

Two Primer Method: Primers such as



are prepared by placing the quencher on the 5' end of the reverse primer for both Bgl II Bcg-1. The Bgl II forward primer is 21bases long with the fluorophore on the 14th base (thymine) from the 5'end and the Bcg-1 forward primer is 25 bases long and with the fluorophore on the 22nd base (thymine) from the 5' end. The quencher is Iowa Black 1.0 and the fluorophore is Fluorescein dT. The reverse and forward Bgl II primers are designed to produce a 42bp amplicon cut by Bgl II into 19bp and 23bp fragments when the V599E mutation is present but remain a 42bp oligonucleotide when the gene is wildtype. The Bcg-1 primers are designed to produce a 53bp amplicon cut by Bcg-1 into 13bp, 34bp, and 6bp fragments when the Q61R mutation is present but remain a 53bp oligonucleotide when the gene is wildtype. The fluorophore is bound within the forward primer, and not at the 5' end, to bring the fluorophore and the quencher to within 100 angstroms of each other.

Fluorophore-Biotin Method: Primers such as



are designed by placing a biotin molecule on the 5' end of the forward primer and a fluorophore on the 5' end of the reverse primer. The fluorophore is 6-Carboxyfluorescein (IDT Technologies). The Bgl II primers are designed to produce a 500bp amplicon cut by Bgl II into 40bp and 460bp fragments if the V599E *B-RAF* mutation is present. The Bcg-1 primers will produce a 500bp amplicon cut by Bcg-1 into 22bp, 34bp, and 444bp fragments if the Q61R *N-RAS* mutation is present.

Initial PCR Reaction and Restriction Enzyme Digestion: The forward and reverse primers are used in a 25 µl PCR reaction at a concentration of 5 picomolar per microliter of each primer. The PCR reaction is carried out using a standard Taq polymerase kit (MasterTaq Kit, Eppendorff) The reaction is performed as a hot-start at 94°°C and commences with a preliminary denaturing step at 94°C for 5 minutes. 40 cycles follow with a denaturing step at 94°C for 45 seconds, an annealing step at 62°C (Bgl II primers) or 55°C (Bcg-1 primers) for 30 seconds, and an elongation step at 72°C for 1 minute. A final step of 72°C for 5 minutes is followed by storage of the PCR product at 4°C until restriction enzyme digestion. Electrophoresis on the PCR product using a 15% ethidium bromide-stained polyacrylamide gel to confirms that the PCR reaction was successful. Next 10 µl of the PCR product is digested in a 20µl reaction containing 1 µl of the restriction enzyme (either Bgl II or Bcg-1, New England Biolabs). The digestion is carried out at 37°C for two hours and the resultant products are run on a 15% ethidium bromide-stained polyacrylamide gel to confirm enzyme digestion. Southern blotting is performed to increase sensitivity of detection. Southern blots are performed on restriction digests of the PCR fragments as follows: PCR reaction mixtures (10 µl) are separated on 3% agarose gels, blotted by capillary transfer to nylon membranes using 10X SSC as the transfer buffer, fixed to the membranes using ultraviolet crosslinking, and then hybridized to the wild type 270 bp B-raf PCR fragment

(Figure 2) labeled with ^{32}P by random priming, in Church buffer at 65° C overnight. The blots are washed serially in 2X, 1X, 0.5X, and 0.2X SSC, with the final wash being 2X SSC at 65°C for 30 minutes, after which they are exposed to Kodak XAR-5 X-ray film with an intensifying screen at –80° C for 4 to 24 hours, depending upon the experiment.

Fluorescent Detection of Restriction Enzyme Digestion: A standard laboratory fluorometer to detect the fluorescent signal is used. For the dual-labeled primer and two-primer methods the restriction enzyme reaction mix is assembled as before produce a 25 µl reaction using 5 µl of PCR product and 1µl of either Bcg-1 or Bgl II. The reaction mix is incubated at 37°C for 2 hours. The fluorescence is measured pre-digestion and at the end of the two hour digestion. For the fluorophore-biotin method the entire 25µl PCR reaction mix from the initial PCR reaction is placed in a clean 0.5ml microcentrifuge tube and 0.3ml of streptavidin-coated metallic beads (MegneSphere Magnetic Separation Beads, Promega, Madison, WI) are added. The mixture is incubated at room temperature for 2 minutes and then the 0.5 ml tube is placed in the magnetic stand to remove the beads from solution. The supernatant is removed and the bead/amplicon complex washed 3 times with phosphate buffered saline (PBS). One microliter of restriction enzyme (Bgl II or Bcg-1) is added, with the appropriate buffer, to the bead-amplicon complex to form a 50 µl reaction, which is incubated at 37°C for 2 hours. The bead-amplicon complex is removed from solution using the magnetic stand and the supernatant removed for fluorescent detection.

Use of Third Oligonucleotide in Two-Primer Method: The initial PCR reaction as described above but before proceeding to the fluorescent detection phase the biotin-labeled third oligo is added to the amplicon/PCR mix at 5 times the initial primer concentration. The third oligo is added to the amplicon/PCR mix at room temperature and the temperature of the reaction raised to 52°C for 5 minutes. The reaction temperature is lowered to 20°C over 30 minutes and then mixed with metallic beads coated with strepavidin (Promega), after which it is incubated at room temperature for 10 minutes and then the metallic beads will be removed from solution using a magnetic stand (Promega). The supernatant will be removed and used in the fluorescent detection reaction as outlined above.

While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made thereto without

departing from the scope and spirit of the present invention, as set forth in the following claims.